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NO:21)

wherein (Chel) is a radiometal-binding moiety.

REMARKS

I. INTRODUCTION

Claim 24 has been amended to delete non-elected subject matter. Applicants reserve the right to file one or more divisional applications covering the non-elected subject matter. Claim 41, as well as the specification, was amended to delete duplicate recitations of peptides and to correct typographical errors vis-à-vis the peptides of SEQ ID 14 and 15: the last "T" in the peptides with SEQ ID 14 and 15 should not be underlined and should read (SEQ ID NO:14) AcK(Chel)F_d CFW_d KTCT-OH and AcK(Chel)DF_d CFW_d KTCT-OH, respectively. Entry of the foregoing amendments to the specification and to claims 21, 41 and 44 is respectfully requested. Upon entry of this amendment, claims 24 - 44 will be pending in this application.

II. THE OFFICE ACTION

A. Election/Restriction

Applicants acknowledge that the restriction requirement is still deemed proper, and, as such, it is final. Further, Applicants acknowledge that the Examiner has withdrawn the restriction between non-elected groups VI and VII, since, according to the Examiner, "it is considered that this is one invention."

B. Objections to the Specification

The Examiner objected to the specification because some of the sequences recited on page 28, line 27 to page 29, line 22 are repeated. Applicants have deleted sequences that are repeated, at the pages noted by the Examiner. As such, reconsideration and withdrawal of the objection is respectfully requested.

C. Rejections based on 35 U.S.C. § 112, First Paragraph-Written Description

Claims 24 - 40 and 42 - 44 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description. Applicants respectfully traverse this ground for rejection.

MPEP Section 2163 provides that the analysis of whether the specification complies with the written description requirement calls for the examiner to compare the scope of the claim with the scope of the description to determine whether applicant has demonstrated possession of the claimed invention. Such a review is conducted from the standpoint of one of skill in the art at the time the application was filed (see, e.g., *Wang Labs. v. Toshiba Corp.*, 993 F.2d 858, 865, 26 USPQ2d 1767, 1774 (Fed. Cir. 1993)) and should include a determination of the field of the invention and the level of skill and knowledge in the art. The guidelines in Section 2163 themselves provide that "***[g]enerally, there is an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement.***" Information which is well known in the art need not be described in detail in the specification. See, e.g., *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986).

Applicants direct the Examiner's attention to page 15, lines 26 to 31, it is clear that Applicants where in possession of the claimed invention; that is, a method of treating a tumor using the claimed radiolabeled peptides. Applicants submit herewith journal articles that show that, at the time the application was filed, the level of skill in the art to which the invention pertains was such that it is not necessary for Applicants to include a great deal of detail in the specification to demonstrate that they were in possession of the invention. At the time the application was filed, it was known that peptides could be used in radionuclide therapy to treat tumors.

de Jong *et al.*, *Cancer Research 58*: 437-441 (1998) (EXHIBIT A)

de Jong *et al.* demonstrated that ^{111}In-labeled somatostatin analogs showed high and specific binding *in vitro* to somatostatin receptors in mouse pituitary AtT20 tumor cell membranes. de Jong *et al.* also showed that all of the compounds they

evaluated, namely, [DTPA^0]octreotide, [DTPA^0,Tyr^3]octreotide, [DTPA^0,D-Tyr^1]octreotide, [DTPA^0,Tyr^3]octreotate, and [DOTA^0,Tyr^3]octreotide, showed specific internalization in rat pancreatic tumor cells. In addition, de Jong *et al.* showed that these results translated to *in vivo* models. For example, biodistribution studies showed that radioactivity in the octreotide-binding, receptor expressing tissues and tumor-to-blood ratios were significantly higher when [^{111}In-DTPA^0,Tyr^3]octreotide, [^{111}In-DTPA^0,Tyr^3]octreotate, and [^{111}In-DOTA^0,Tyr^3]octreotide were used than when [^{111}In-DTPA^0]octreotide was used. Finally, de Jong *et al.* characterize radiolabeled [DTPA^0,Tyr^3]octreotide, and especially [DTPA^0,Tyr^3]octreotate and their DOTA-coupled counterparts as "most promising for scintigraphy and radionuclide therapy of [somatostatin] receptor-positive tumors in humans.

Lewis *et al.*, *J. Med. Chem. 42*: 1341-1347 (1999) (EXHIBIT B)

In a study which illustrated the structure activity relationship of various somatostatin analogs related to those described by de Jong *et al.* (*supra*), Lewis *et al.* compared the *in vitro* binding, *in vitro* tumor cell uptake, and *in vivo* distribution of [^{64}Cu-TETA,Tyr^3]octreotide and [^{64}Cu-TETA]octreotate with that of [^{64}Cu-TETA,Tyr^3]octreotate and [^{64}Cu-TETA]octreotide. Applicants note that they have used the same type of nomenclature used in the de Jong *et al.* to describe the peptides of Lewis *et al.* Lewis *et al.* demonstrated that, while all of these peptides displayed affinity for somatostatin receptors on CA20948 rat pancreatic tumor membranes, [^{64}Cu-TETA]octreotate and [^{64}Cu-TETA,Tyr^3]octreotate showed the highest affinity for the receptors. Biodistributions in CA20948 tumor-bearing rats showed receptor mediated uptake of the ^{64}Cu-labeled peptides in somatostatin-rich tissues, including the pituitary adrenals, pancreas, and tumor. Lewis *et al.* found that [^{64}Cu-TETA,Tyr^3]octreotate exhibited the highest tumor uptake of all of the peptides studied.

Lewis *et al. Clinical Cancer Research 5*: 3608-3616 (1999) (EXHIBIT C)

In this study, Lewis *et al.* mention a previous study which showed that [^{64}Cu-TETA]octreotide significantly exhibited the growth of somatostatin receptor-positive CA20948 rat pancreatic tumors in Lewis rats. Anderson *et al.*, *J. Nucl. Med. 39*: 1944-

1951 (1998). In the current study, Lewis *et al.* found that a single dose of 15 mCi of [^{64}Cu-TETA,Tyr^3]octreotate was shown to be more effective in reducing tumor burden than the same dose of [^{64}Cu-TETA]octreotide. Lewis *et al.* also found that in multiple dose experiments, complete regression of tumors was observed for all rats treated with 3 x 20 mCi of [^{64}Cu-TETA,Tyr^3]octreotate; with no palpable tumors for approximately 10 days. Lewis *et al.* found that the mean survival time of the rats was nearly twice that of controls.

The three journal articles described above show the level of skill in the art at the time the application was filed. The ordinary skilled artisan would therefore know how to use radiolabeled peptides, such as those disclosed and claimed in the present application, to treat tumors. Applicants assert, therefore, that it is not necessary to describe in the specification information which is well known in the art such as how the claimed peptides would be used to treat tumors. *See, e.g., Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986). Accordingly, the instant specification not only complies with the written description requirement, but it also demonstrates that Applicants had possession of the claimed invention.

Reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph is respectfully requested.

C. Rejections Based on 35 U.S.C. § 112, Second Paragraph

Claims 24 - 44 stand rejected under 35 U.S.C. § 112, second paragraph, as being allegedly indefinite. Applicants respectfully traverse this ground for rejection.

1. Rejection based on the recitation of two statutory subject matters

The Examiner stated that claim 24 is confusing in that it covers two statutory subject matters: method of using and method of making.

Applicants are not aware of any provision in the MPEP or U.S. Code that would preclude Applicants from claiming a method of using the claimed radiolabeled peptides to treat tumors, where the radiolabeled peptides are characterized by the way

they are made. Further, Applicants are not aware of any provision in the MPEP or U.S. Code which would render such a claim indefinite. Applicants respectfully urge the Examiner to provide the section in the MPEP or statutory basis which would render such a claim indefinite. In the absence of such bases, Applicants assert that claim 24 is definite.

Reconsideration and withdrawal of the rejection of claim 24 under 35 U.S.C. § 112, second paragraph is respectfully requested.

2. Rejection Based on lack of clarity with respect to the term "together" in claim 24

The Examiner stated that it is not clear whether the administration of the radiolabeled peptide "together" with the carrier is as a unit or two separate components. Applicants have amended claim 24 to recite that a radiolabeled peptide **and** a pharmaceutically acceptable carrier are administered to treat a tumor. Applicants have amended claim 24, as such, to convey that the two components are added as a unit and not as separate components.

Reconsideration and withdrawal of the rejection is respectfully requested.

3. Rejection based on the term "lower" in claim 24

The Examiner alleges that the term "lower" in "lower alkyl" is a relative term. The Examiner has suggested that Applicants recite the number of carbon atoms contained therein. Likewise, the Examiner suggests that Applicants recite the number of carbon atoms contained in the aryl and cycloalkyl groups. Finally, the Examiner alleges that the term "substituted" is indefinite as to the kind, number and position of each of the substituents presented therein.

Applicants appreciate the Examiner's kind suggestions with respect to amending claim 24 to recite the number of carbon atoms in the lower alkyl, aryl and cycloalkyl groups. Applicants provide, however, that this is not necessary, as the specification clearly describes the number of carbon atoms in each of the aforementioned groups at page 11, lines 7 - 23. Thus, the terms lower alkyl, aryl and cycloalkyl are indeed definite, as they are clearly described in the specification at the

aforementioned page and line numbers.

Applicants respectfully disagree with the Examiner's allegations that the term "substituted" is indefinite as to the kind of substituents contemplated.. The kinds of substituents contemplated are described at page 11, lines 24 - 34. With respect to the "number and position of each of the substituents," Applicants provide that the skilled artisan would recognize, for example, that an aryl group could contain up to 5 substituents; in theory one at each ortho and meta position and one at the para position. Thus, Applicants assert that the term "substituted" is definite vis-à-vis the kind, number and positions of each of the substituents presented therein.

Reconsideration and withdrawal of the rejection is respectfully requested.

4. Rejection based on the phrases "a protecting group that can be removed under the conditions of peptide synthesis" and "then contacting said solution with a radionuclide and recovering the radiolabeled peptide" in claim 24

The Examiner stated that in claim 24 it is not clear whether the compound administered is in protected or non-protected form.

Claim 24 recites that R_1, R_2, and R_3 are independently a number of substituents, one of which is a protecting group that can be removed under the conditions of peptide synthesis. Applicants assert that the skilled artisan would know this to mean that, for example, when R_1, R_2 and R_3 are anything but "a protecting group that can be removed under the conditions of peptide synthesis," then the peptide is unprotected. Contrariwise, when one of R_1, R_2 and R_3 is "a protecting group that can be removed under the conditions of peptide synthesis," then the peptide is protected. Thus, Applicants assert that the phrase "a protecting group that can be removed under the conditions of peptide synthesis," is clear and definite.

In making the rejection based on the phrases "a protecting group that can be removed under the conditions of peptide synthesis" and "then contacting said solution with a radionuclide and recovering the radiolabeled peptide," the Examiner again raises the issue that both of these phrases relate to a method of making and not using the compound. Once again, Applicants provide that they are not aware of any provision in

the MPEP or U.S. Code that would preclude Applicants from claiming a method of using the claimed radiolabeled peptides to treat tumors, where the radiolabeled peptides are characterized by the way they are made.

Reconsideration and withdrawal of the rejection of claim 24 under 35 U.S.C. § 112, second paragraph is respectfully requested.

5. Rejection of claim 44

The Examiner asserts that the recitation of the term "protein" in claim 44 broadens claim 42, which recites the term "peptide." In light of the cancellation ofs claim 44, Applicants assert that the rejection has been overcome.

Reconsideration and withdrawal of the rejection is respectfully requested.

6. Rejection of claim 41

The Examiner states that claim 41 contains sequences that are duplicative. In light of the amendments to claim 41, Applicants assert that the rejection has been rendered moot. Specifically, Applicants have deleted those sequences from claim 41 that are duplicative.

Reconsideration and withdrawal of the rejection is respectfully requested.

III. CONCLUSION

The claimed invention is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Respectfully submitted,

October 31, 2002

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Version with Markings to Show Changes Made

IN THE SPECIFICATION:

Page 8, line 27 to Page 9, line 22:

In accordance with another aspect of the invention there are provided peptides having a structure selected from the group consisting of:

(Chel)γAbuNleDHF$_d$ RWK-NH_2, (SEQ ID NO:1)

(Chel)γAbuHSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2, (SEQ ID NO:2)

KPRRPYTDNYTRLRK(Chel)QMAVKKYLNSILN-NH_2, (SEQ ID NO:3)

(Chel)γAbuVFTDNYTRLRKQMAVKKYLNSILN-NH_2,

(Chel)γAbuYTRLRKQMAVKKYLNSILN-NH_2, (SEQ ID NO:4)

HSDAVFTDNYTRLRK(Chel)QMAVKKYLNSILN-NH_2, (SEQ ID NO:5)

(SEQ ID NO:6) <GHWSYK(Chel)LRPG-NH_2, <GHYSLK(Chel)WKPG-NH_2, (SEQ ID NO:7)

AcNal_d Cpa_d W_d SRK_d (Chel)LRPA_d -NH_2, (SEQ ID NO:8)

(SEQ ID NO:9) (Chel)γAbuSYSNleDHF$_d$ RWK-NH_2, ~~(Chel)γAbuNleDHF$_d$ RWK-NH_2, (SEQ ID NO:1)~~

~~(Chel)NleDHF$_d$ RWK-NH_2, (SEQ ID NO:1)~~

Ac-HSDAVFTENYTKLRK(Chel)QNleAAKKYLNDLKKGGT-NH_2, (SEQ ID NO:10)

~~(Chel)γAbuHSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2, (SEQ ID NO:2)~~

~~(Chel)γAbuVFTDNYTRLRKQMAVKKYLNSILN-NH_2, (SEQ ID NO:4)~~

~~(SEQ ID NO:1) (Chel)γAbuNleDHF$_d$ RWK-NH_2[e], <GHWSYK(Chel)LRPG-NH_2, (SEQ ID NO:6)~~

~~(SEQ ID NO:7) <GHYSLK(Chel)WKPG-NH_2, AcNal_d CPa_d W_d SRK_d (Chel)LRPA_d-NH_2, (SEQ ID NO:8)~~

~~(SEQ ID NO:11) <GHYSYLK(Chel)WKPG-NH_2, <GHYSLK(Chel)WKPG-NH_2, (SEQ ID NO:9)~~

(SEQ ID NO:12) Nal_d Cpa_d W_d SRK_d (Chel)WKPG-NH_2, <GHWSYK_d (Chel)LRPG-NH_2, (SEQ ID NO:13)

~~AcNal_d Cpa_d W_d SRK_d (Chel)LRPA_d-NH_2, (SEQ ID NO:8) AcNal_d Cpa_d W_d SRK_d (Chel)LRPA_d-NH_2, (SEQ ID NO:8)~~

~~(SEQ ID NO:8) AcNal$_d$ Cpa$_d$ W$_d$ SRK$_d$ (Chel)LRPA$_d$ NH$_2$, <GHWSYK(Chel)LRPG-NH$_2$, (SEQ ID NO:6)~~

(SEQ ID NO:14) AcK(Chel)F$_d$ CFW$_d$ KTC~~T~~T-OH, AcK(Chel)DF$_d$ CFW$_d$ KTC~~T~~T-OH, (SEQ ID NO:15)

(SEQ ID NO:14) AcK(Chel)F$_d$ CFW$_d$ KTCT-ol, AcK(Chel)DF$_d$ CFW$_d$ KTCT-ol, (SEQ ID NO:15)

(SEQ ID NO:16) (Chel)DF$_d$ CFW$_d$ KTCT-OH, K(Chel)DF$_d$ CFW$_d$ KTCT-ol, (SEQ ID NO:15)

(SEQ ID NO:17) K(Chel)KKF$_d$ CFW$_d$ KTCT-ol, K(Chel)KDF$_d$ CFW$_d$ KTCT-OH, (SEQ ID NO:18)

(SEQ ID NO:19) K(Chel)DSF$_d$ CFW$_d$ KTCT-OH, K(Chel)DF$_d$ CFW$_d$ KTCT-OH, (SEQ ID NO:15)

(SEQ ID NO:20) K(Chel)DF$_d$ CFW$_d$ KTCD-NH$_2$, K(Chel)DF$_d$ CFW$_d$ KTCT-NH$_2$, (SEQ ID NO:15)

(SEQ ID NO:18) K(Chel)KDF$_d$ CFW$_d$ KTCT-NHNH$_2$, AcK(Chel)F$_d$ CFW$_d$ KTCT-NHNH$_2$, (SEQ ID NO:14)

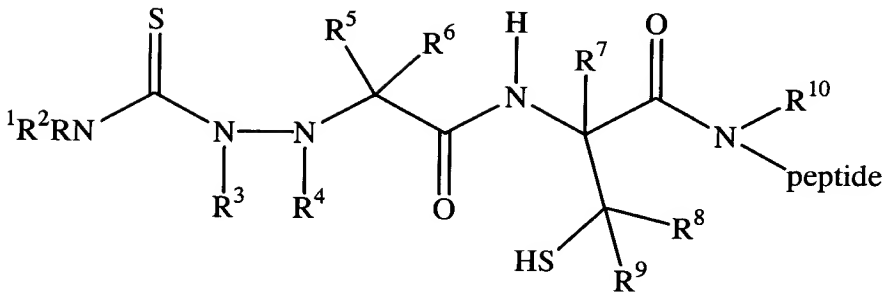
(SEQ ID NO:14) K(Chel)F$_d$ CFW$_d$ KTCT-ol, and F$_d$ CFW$_d$ KTCTK(Chel)-NH$_2$, (SEQ ID NO:21)

wherein (Chel) is a radiometal-binding moiety having the structure set forth above.

IN THE CLAIMS:

24. (Amended) A method of treating a tumor~~, an infectious lesion, a myocardial infaretion, a clot, atherosclerotic plaque, or a normal organ or tissue~~, comprising administering to a human patient a radiolabeled peptide~~, together with~~ and a pharmaceutically acceptable carrier,

wherein said radiolabeled peptide is prepared by contacting a solution of a peptide with stannous ions, wherein said peptide comprises a radiometal-binding moiety comprising the structure:



wherein R^1, R^2, and R^3 independently are selected from the group consisting of H, lower alkyl, substituted lower alkyl, cycloalkyl, substituted cycloalkyl, heterocycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, alkaryl, and a protecting group that can be removed under the conditions of peptide synthesis, provided that at least one of R^1, R^2, or R^3 is H,

R^5, R^7, R^8, R^9 and R^{10} independently are selected from the group consisting of H, lower alkyl, substituted lower alkyl, aryl, and substituted aryl, and R^8 and R^9 together or R^7 and R^9 together may form a cycloalkyl or substituted cycloalkyl ring,

R^4 and R^6 together form a direct bond or are independently selected from the group consisting of lower alkyl, substituted lower alkyl, aryl, and substituted aryl, and wherein NR^{10} is located at the N-terminus of said peptide, or is located on an amino acid side chain of said peptide,

and then contacting said solution with a radionuclide and recovering the radiolabeled peptide.

41. (Amended) A method according to claim 24, wherein said peptide is selected from the group consisting of:

(Chel)γAbuNleDHF$_d$ RWK-NH_2, (SEQ ID NO:1)

(Chel)γAbuHSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2, (SEQ ID NO:2)

KPRRPYTDNYTRLRK(Chel)QMAVKKYLNSILN-NH_2, (SEQ ID NO:3)

(Chel)γAbuVFTDNYTRLRKQMAVKKYLNSILN-NH_2,

(Chel)γAbuYTRLRKQMAVKKYLNSILN-NH_2, (SEQ ID NO:4)

HSDAVFTDNYTRLRK(Chel)QMAVKKYLNSILN-NH_2, (SEQ ID NO:5)

(SEQ ID NO:6) <GHWSYK(Chel)LRPG-NH_2, <GHYSLK(Chel)WKPG-NH_2, (SEQ ID NO:7)

AcNal$_d$ Cpa$_d$ W$_d$ SRK$_d$ (Chel)LRPA$_d$ -NH$_2$, (SEQ ID NO:8)

(SEQ ID NO:9) (Chel)γAbuSYSNleDHF$_d$ RWK-NH$_2$, Ac-HSDAVFTENYTKLRK(Chel)QNleAAKKYLNDLKKGGT-NH$_2$, (SEQ ID NO:10)

(SEQ ID NO:12) Nal$_d$ Cpa$_d$ W$_d$ SRK$_d$ (Chel)WKPG-NH$_2$, <GHWSYK$_d$ (Chel)LRPG-NH$_2$, (SEQ ID NO:13)

(SEQ ID NO:14) AcK(Chel)F$_d$ CFW$_d$ KTCT-OH, AcK(Chel)DF$_d$ CFW$_d$ KTCT-OH, (SEQ ID NO:15)

(SEQ ID NO:14) AcK(Chel)F$_d$ CFW$_d$ KTCT-ol, AcK(Chel)DF$_d$ CFW$_d$ KTCT-ol, (SEQ ID NO:15)

(SEQ ID NO:16) (Chel)DF$_d$ CFW$_d$ KTCT-OH, K(Chel)DF$_d$ CFW$_d$ KTCT-ol, (SEQ ID NO:15)

(SEQ ID NO:17) K(Chel)KKF$_d$ CFW$_d$ KTCT-ol, K(Chel)KDF$_d$ CFW$_d$ KTCT-OH, (SEQ ID NO:18)

(SEQ ID NO:19) K(Chel)DSF$_d$ CFW$_d$ KTCT-OH, K(Chel)DF$_d$ CFW$_d$ KTCT-OH, (SEQ ID NO:15)

(SEQ ID NO:20) K(Chel)DF$_d$ CFW$_d$ KTCD-NH$_2$, K(Chel)DF$_d$ CFW$_d$ KTCT-NH$_2$, (SEQ ID NO:15)

(SEQ ID NO:18) K(Chel)KDF$_d$ CFW$_d$ KTCT-NHNH$_2$, AcK(Chel)F$_d$ CFW$_d$ KTCT-NHNH$_2$, (SEQ ID NO:14)

(SEQ ID NO:14) K(Chel)F$_d$ CFW$_d$ KTCT-ol, and F$_d$ CFW$_d$ KTCTK(Chel)-NH$_2$, (SEQ ID NO:21)

wherein (Chel) is a radiometal-binding moiety.~~(Chel)γAbuNleDHF$_d$RWK-NH$_2$, (Chel)γAbuHSDAVFTDNYTRLRKQMAVKKYLNSILN-NH$_2$, KPRRPYTDNYTRLRK(Chel)QMAVKKYLNSILN-NH$_2$, (Chel)γAbuVFTDNYTRLRKQMAVKKYLNSILN-NH$_2$, (Chel)γAbuYTRLRKQMAVKKYLNSILN-NH$_2$, HSDAVFTDNYTRLRK(Chel)QMAVKKYLNSILN-NH$_2$, <GHWSYK(Chel)LRPG-NH$_2$, <GHYSLK(Chel)WKPG-NH$_2$, AcNal$_d$Cpa$_d$W$_d$SRK$_d$(Chel)LRPA$_d$-NH2 , (Chel)γAbuSYSNleDHF$_d$RWK-NH$_2$, (Chel)γAbuNleDHF$_d$RWK-NH$_2$, (Chel)NleDHF$_d$RWK-NH$_2$, Ac-HSDAVFTENYTKLRK(Chel)QNleAAKKYLNDLKKGGT-NH$_2$,~~

~~(Chel)γAbuHSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2;~~

~~(Chel)γAbuVFTDNYTRLRKQMAVKKYLNSILN-NH_2;~~

~~(Chel)γAbuNle~~DHF$_d$~~RWK-NH_2~~[e]~~;~~ <GHWSYK(Chel)LRPG-NH_2;~~

~~<GHYSLK(Chel)WKPG-NH_2; AcNal$_d$Cpa$_d$W$_d$SRK$_d$(Chel)LRPA$_d$-NH_2;~~

~~<GHYSYLK(Chel)WKPG-NH_2; <GHYSLK(Chel)WKPG-NH_2;~~

~~Nal$_d$Cpa$_d$W$_d$SRK$_d$(Chel)WKPG-NH_2; <GHWSYK$_d$(Chel)LRPG-NH_2;~~

~~AcNal$_d$Cpa$_d$W$_d$SRK$_d$(Chel)LRPA$_d$-NH_2; AcNal$_d$Cpa$_d$W$_d$SRK$_d$(Chel)LRPA$_d$-NH_2;~~

~~AcNal$_d$Cpa$_d$W$_d$SRK$_d$(Chel)LRPA$_d$-NH_2; <GHWSYK(Chel)LRPG-NH_2;~~

~~AcK(Chel)F$_d$CFW$_d$KTCT-OH; AcK(Chel)DF$_d$CFW$_d$KTCT-OH;~~

~~AcK(Chel)F$_d$CFW$_d$KTCT-ol; AcK(Chel)DF$_d$CFW$_d$KTCT-ol; (Chel)DF$_d$CFW$_d$KTCT-OH; K(Chel)DF$_d$CFW$_d$KTCT-ol; K(Chel)KKF$_d$CFW$_d$KTCT-ol;~~

~~K(Chel)KDF$_d$CFW$_d$KTCT-OH; K(Chel)DSF$_d$CFW$_d$KTCT-OH;~~

~~K(Chel)DF$_d$CFW$_d$KTCT-OH; K(Chel)DF$_d$CFW$_d$KTCD-NH_2;~~

~~K(Chel)DF$_d$CFW$_d$KTCT-NH_2; K(Chel)KDF$_d$CFW$_d$KTCT-$NHNH_2$;~~

~~AcK(Chel)F$_d$CFW$_d$KTCT-$NHNH_2$; K(Chel)F$_d$CFW$_d$KTCT-ol; and~~

~~F$_d$CFW$_d$KTCTK(Chel)-NH_2;~~

~~wherein (Chel) is said radiometal-binding moiety.~~

[CANCER RESEARCH 58, 437–441, February 1, 1998]

Comparison of ^{111}In-labeled Somatostatin Analogues for Tumor Scintigraphy and Radionuclide Therapy[1]

Marion de Jong,[2] Wout A. P. Breeman, Willem H. Bakker, Peter P. M. Kooij, Bert F. Bernard, Leo J. Hofland, Theo J. Visser, Ananth Srinivasan, Michelle A. Schmidt, Jack L. Erion, Joseph E. Bugaj, Helmut R. Mäcke, and Eric P. Krenning

Departments of Nuclear Medicine [M. d. J., W. A. P. B., W. H. B., P. P. M. K., B. F. B., E. P. K.] and Internal Medicine III [L. J. H., T. J. V., E. P. K.], University Hospital Dijkzigt, 3015 GD Rotterdam, the Netherlands; Mallinckrodt Medical, St. Louis, Missouri 63134 [A. S., M. A. S., J. L. E., J. E. B.]; and Department of Nuclear Medicine, Kantonspital Basel, CH-4031 Basel, Switzerland [H. R. M.]

ABSTRACT

We evaluated the following ^{111}In-labeled somatostatin (SS) analogues (diethylenetriaminepentaacetic acid, DTPA; tetraazacyclododecanetetraacetic acid, DOTA): [DTPA^0]octreotide, [DTPA^0,Tyr^3]octreotide, [DTPA^0,D-Tyr^1]octreotide, [DTPA^0,Tyr^3]octreotate [Thr(ol) in octreotide replaced with Thr], and [DOTA^0,Tyr^3]octreotide, *in vitro* and *in vivo*.

***In vitro*, all compounds showed high and specific binding to SS receptors in mouse pituitary AtT20 tumor cell membranes, and IC_{50}s were in the nanomolar range. Furthermore, all compounds showed specific internalization in rat pancreatic tumor cells; uptake of [^{111}In-DTPA^0,Tyr^3]octreotate was the highest of the compounds tested, and that of [^{111}In-DTPA^0,D-Tyr^1]octreotide was the lowest. Biodistribution experiments in rats showed that, 4, 24, and 48 h after injection of [^{111}In-DTPA^0,Tyr^3]octreotide, [^{111}In-DTPA^0,Tyr^3]octreotate, and [^{111}In-DOTA^0,Tyr^3]octreotide, radioactivity in the octreotide-binding, receptor-expressing tissues and tumor-to-blood ratios were significantly higher than those after injection of [^{111}In-DTPA^0]octreotide. Uptake of [^{111}In-DTPA^0,Tyr^3]octreotate in the target organs was also, *in vivo*, the highest of the radiolabeled peptides tested, whereas that of [^{111}In-DTPA^0,D-Tyr^1]octreotide was the lowest. Uptake of [^{111}In-DTPA^0,Tyr^3]octreotide, [^{111}In-DTPA^0,Tyr^3]octreotate, and [^{111}In-DOTA^0,Tyr^3]octreotide in target tissues was blocked by >90% by 0.5 mg of unlabeled octreotide, indicating specific binding to the octreotide receptors. Blockade of [^{111}In-DTPA^0,D-Tyr^1]octreotide was >70%. In conclusion, radiolabeled [DTPA^0,Tyr^3]octreotide and, especially, [DTPA^0,Tyr^3]octreotate and their DOTA-coupled counterparts are most promising for scintigraphy and radionuclide therapy of SS receptor-positive tumors in humans.**

INTRODUCTION

Radiolabeled tumor receptor-binding peptides can be used for *in vivo* scintigraphic imaging. An example is SS,[3] which binds to its receptors on tumors of neuroendocrine origin (1). The native peptide, however, is susceptible to very rapid enzymatic degradation (2) and is, therefore, not useful for *in vivo* application. Therefore, more stable synthetic SS analogues have been developed; *e.g.*, the octapeptide octreotide (Fig. 1; Ref. 3). Because octreotide cannot be radiolabeled easily with a γ-emitting radionuclide, Tyr^3-octreotide was developed, allowing radioiodination of the molecule (Fig. 1). This compound, radiolabeled with ^{125}I or ^{123}I, was the first used in *in vitro* SS receptor studies (4), tumor scintigraphy in animals (4), and in humans (1, 5). [^{111}In-DTPA^0]octreotide, consisting of the octapeptide octreotide and the chelator DTPA (Fig. 1), enabling radiolabeling with a radiometal like ^{111}In, was the next SS analogue to be synthesized for scintigraphy of SS receptor-positive lesions *in vivo*. We have described its advantages over radioiodinated Tyr^3-octreotide and its use for scintigraphic imaging of SS receptor-positive lesions (6, 7).

A new and fascinating application is the use of radiolabeled octreotide for radionuclide therapy. Promising results with regard to tumor growth inhibition have been reported in humans using [^{111}In-DTPA^0]octreotide (8). A β^- particle emitter, such as ^{90}Y, may, in certain cases, appear more suitable for this purpose than the Auger electron emitter ^{111}In. However, ^{90}Y-DTPA is unstable, resulting in hematopoietic toxicity *in vivo*; therefore, Tyr^3-octreotide has been derivatized with the DOTA chelator (Fig. 1), enabling stable radiolabeling with ^{90}Y and ^{111}In. (Pre)clinical studies with [DOTA^0,Tyr^3]octreotide showed favorable biodistribution and tumor uptake characteristics (9–11).

The success of the therapeutic strategy relies upon the amount of radioligand, which can be concentrated within tumor cells, and this will, among other things, be determined by the rates of internalization, degradation, and recycling of both ligand and receptor. We have evaluated and compared the different mentioned ^{111}In-chelator-peptide constructs, and we have also studied some new, recently synthesized SS analogues, with regard to binding to octreotide receptors on mouse pituitary tumor cell membranes and internalization in rat pancreatic tumor cells. Furthermore, biodistribution in tumor-bearing rats was investigated *in vivo*. The newly synthesized analogues tested were [DTPA^0,D-Tyr^1]octreotide and [DTPA^0,Tyr^3]octreotate (structures shown in Fig. 1).

MATERIALS AND METHODS

Labeling of Octreotide Derivatives. [DTPA^0]octreotide and $^{111}InCl_3$ were provided by Mallinckrodt Medical (Petten, the Netherlands), and octreotide was supplied by Sandoz (Basel, Switzerland). [DOTA^0,Tyr^3]octreotide was synthesized by H. R. M., and [DTPA^0,D-Tyr^1]octreotide, [DTPA^0,Tyr^3]-octreotide, and [DTPA^0,Tyr^3]octreotate were synthesized by A. S. ^{111}In labeling of the DTPA-analogues was as described for [DTPA^0]octreotide (12), and ^{111}In-labeling of [DOTA^0,Tyr^3]octreotide (9) and ^{125}I-labeling of [Tyr^3]octreotide (4) were performed as described.

***In Vitro* Receptor Binding Studies.** Receptor binding assays were carried out using [^{125}I-Tyr^3]octreotide (2200 Ci/mmol) as radioligand using mouse AtT20 pituitary tumor cell membranes (13).

Internalization. AR42J cells were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), CA20948 cells were grown in DMEM (Life Technologies, Inc.), and ARO cells were grown in DMEM/F12 (Life Technologies, Inc.); for all cell lines, medium was supplemented with 2 mM glutamine and 10% FCS. Before the experiment, subconfluent cell cultures were transferred to six-well plates.

The binding of the radiolabeled peptides to tumor cells and subsequent internalization were studied essentially as described (14). In short, before the experiments, cells were washed, and incubation was started by addition of 1 ml of internalization medium/well (culture medium without FCS but with 1% BSA) with 80 kBq of peptide (0.1 nM concentration). Cells were incubated at 37°C for indicated periods of time. To determine nonspecific internalization, cells were incubated with an excess unlabeled octreotide (0.1 μM). Cellular

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[3] The abbreviations used are: SS, somatostatin; DTPA, diethylenetriaminepentaacetic acid; DOTA, tetraazacyclododecanetetraacetic acid; AUC, area under the curve; %ID, percentage injected dose.

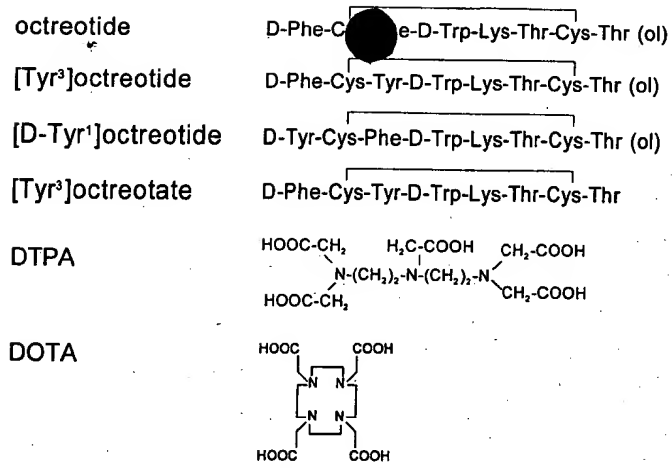

Fig. 1. Structures of octreotide, D-Tyr¹-octreotide, Tyr³-octreotide, Tyr³-octreotate, DTPA, and DOTA.

Table 1 *IC_{50} of unlabeled peptides*

Binding of [^{125}I-Tyr^3]octreotide to mouse AtT20 pituitary cell membranes. Results are means of triplicate measurements in a representative experiment.

Unlabeled peptide	IC_{50} (nM)
[DTPA^0]octreotide	3
[DTPA^0,Tyr^3]octreotide	3.2
[DTPA^0,D-Tyr^1]octreotide	6.3
[DTPA^0,Tyr^3]octreotate	3.6
[DOTA^0,Tyr^3]octreotide	0.6

uptake was stopped by removing medium from the cells and washing with 2 ml of ice-cold PBS. To discriminate between internalized and noninternalized (surface-bound) radiopharmaceuticals, intact cells were incubated with 1 ml of 20 mM sodium acetate. The internalized and noninternalized fractions were determined by measuring radioactivity in a LKB-1282-Compugammasystem. The internalized fraction was expressed as percentage of the applied dose per mg of cellular protein. The latter was determined using a commercially available kit (Bio-Rad, Veenendaal, the Netherlands).

***In Vivo* Tissue Distribution.** Animal experiments were performed in compliance with regulations of our institution and with generally accepted guidelines governing such work. Male Lewis rats, bearing the CA20948 pancreatic tumor or Wistar male rats (200–250 g) were used in the experiments. Rats were injected under ether anesthesia with 3 MBq (0.5 μg) of ^{111}In-labeled peptide in 200 μl of saline into the dorsal vein of the penis. To determine nonspecific binding of the radiopharmaceutical, a separate group of rats was injected s.c. with 0.5 mg of octreotide in 1 ml of 0.05 M acetic acid in saline, 30 min before injection of the radiolabeled peptide. At the indicated time points, rats were sacrificed under ether anesthesia. Organs and blood were collected, and the radioactivity in these samples was determined using a LKB-1282-Compugammasystem. Statistical evaluation was performed using one-way ANOVA, followed by comparison among class means and Student's *t* test, corrected for multiple pairwise comparisons between means.

RESULTS

Radiolabeling. ^{111}In-labeling efficiency of the different peptides and radioiodination efficiency of [Tyr^3]octreotide ranged from 97 to 100%.

***In Vitro* Receptor Binding Studies.** Table 1 shows that unlabeled peptides had high and specific binding for the octreotide-binding receptors (mostly sst_2) on AtT20 membranes; the IC_{50} values were all in the nanomolar range. [DOTA^0,Tyr^3]octreotide showed the highest affinity.

***In Vitro* Internalization Studies.** Table 2 shows specific internalization, that is, total internalization corrected for internalization in the presence of a block dose of octreotide of the ^{111}In-labeled peptides in the octreotide receptor-positive rat pancreatic cell lines after a 60-min incubation at 37°C. Data were expressed as percentages of specific internalization of [^{125}I-Tyr^3]octreotide. As is shown, internalized radioactivity of [^{111}In-DTPA^0,Tyr^3]octreotate was the highest of the compounds tested, whereas that of [^{111}In-DTPA^0,D-Tyr^1]octreotide was the lowest.

Tissue Distribution in Rats. Fig. 2 presents radioactivity in octreotide binding receptor-positive (mostly sst_2) organs, including pancreas, adrenals, pituitary, and CA20948 rat pancreatic tumors, 4, 24, and 48 h after injection of the radiolabeled peptides. Uptake in these octreotide receptor-expressing organs at the time points tested was highest for [^{111}In-DTPA^0,Tyr^3]octreotate and lowest for [^{111}In-DTPA^0,D-Tyr^1]octreotide.

Table 3 shows that uptake of ^{111}In-labeled peptides in these octreotide receptor-positive target organs represented mostly specific binding to the octreotide receptors because uptake was decreased to less than 7% of control by pretreatment of the rats with 0.5 mg of unlabeled octreotide, except for [^{111}In-DTPA^0,D-Tyr^1]octreotide, for which uptake was decreased to about 30% of control.

In Table 4, radioactivity in octreotide receptor-negative organs and blood 24 h after injection of the tested ^{111}In-labeled peptides is shown. Clearance from the blood was rapid and comparable for all peptides. The radiolabeled peptides were excreted in the urine very rapidly and mostly intact; over 95% of the excreted radioactivity after 24 h was intact radiolabeled peptide (data not shown). Furthermore, the low uptake of [^{111}In-DTPA^0,Tyr^3]octreotate in the liver is worth mentioning, which is favorable, especially in combination with the rapid blood clearance and high uptake of this compound in the target organs.

The data obtained *in vivo*, as shown in Fig. 2 and Table 4, are summarized in Fig. 3, in which the AUC (h·%ID/g) for each group between 4 and 48 h post injection is shown. The top panel shows that radioactivity/g tissue in this time period was low in blood, liver, and spleen and higher in kidneys, tumor, and octreotide receptor (sst_2)-positive organs. The bottom panel shows the same data but expressed as percentage of the AUC of [^{111}In-DTPA^0]octreotide in the different organs. In the sst_2-negative organs, AUC is comparable for the peptides, except for the low liver AUC of [^{111}In-DTPA^0,Tyr^3]octreotate. In the sst_2-positive organs, the AUC of [^{111}In-DTPA^0,Tyr^3]octreotate is the highest.

DISCUSSION

Compared to ^{123}I-Tyr^3-octreotide, [^{111}In-DTPA^0]octreotide was the preferred analogue for *in vivo* scintigraphy because it has several advantages: general availability, simple one-step method of radiolabeling, longer physiological half-life in plasma, and a more suitable metabolism (5). A new field of application of radiolabeled SS ana-

Table 2 *Comparison of specific internalization of [^{111}In-DTPA^0]octreotide, [^{111}In-DTPA^0,D-Tyr^1]octreotide, [^{111}In-DTPA^0,Tyr^3]octreotide, [^{111}In-DTPA^0,Tyr^3]octreotate, and [^{111}In-DOTA^0,Tyr^3]octreotide after a 60-min incubation at 37°C*

Data for each experiment expressed as percentage of specific [^{125}I-Tyr^3]octreotide internalization (range, 6.5 ± 0.8%–9.2 ± 1.1% dose) tested in the same experiment. Data are the means of those obtained in at least two different experiments in the two octreotide receptor-positive cell lines used (CA20948 and AR42J).

Compound	Mean (SD)
[^{125}I-Tyr^3]octreotide	100 (12)
[^{111}In-DTPA^0]octreotide	8.2 (0.7)[a]
[^{111}In-DTPA^0,D-Tyr^1]octreotide	2.2 (1.1)[a]
[^{111}In-DTPA^0,Tyr^3]octreotide	40.2 (4.5)[a]
[^{111}In-DTPA^0,Tyr^3]octreotate	211.5 (12)[a]
[^{111}In-DOTA^0,Tyr^3]octreotide	14.6 (1.1)[a]

[a] $P < 0.001$ *versus* [^{125}I-Tyr^3]octreotide.

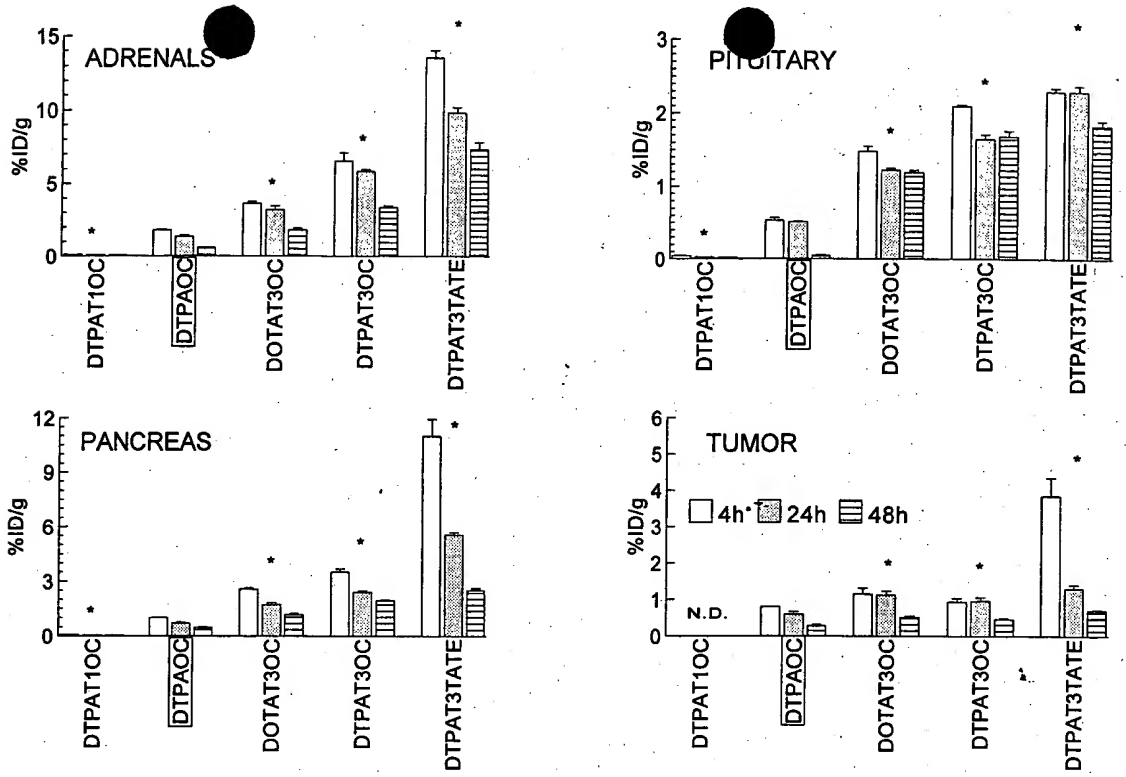

Fig. 2. Radioactivity in octreotide receptor-expressing organs 4, 24, and 48 h after injection of the ^{111}In-labeled peptides in rats. *Columns*, mean %ID/g ($n \geq 6$); *bars*, SE. *ND*, not determined; *DTPAOC* [DTPA^0]octreotide; *DTPAT1OC*, [DTPA^0,D-Tyr^1]octreotide; *DTPAT3OC*, [DTPA^0,Tyr^3]octreotide; *DTPAT3TATE*, [DTPA^0,Tyr^3]octreotate: *DOTAT3OC*, [DOTA^0,Tyr^3]octreotide. *, $P < 0.001$ *versus* [^{111}In-DTPA^0]octreotide, for all time points tested.

Table 3 *Radioactivity in SS receptor-positive organs of octreotide-pretreated rats 24 h after administration of the ^{111}In-labeled peptides*

Labeled compound was injected 30 min after s.c. injection of 0.5 mg of unlabeled octreotide or vehicle (control). Tissue radioactivity in octreotide-pretreated rats is expressed as a percentage of that in controls [for each group $n \geq 6$ mean (SE)].

Treatment	Pituitary	Pancreas	Adrenals	Tumor
[DTPA^{0}octreotide	6.9 (0.7)[a]	3.5 (0.03)[a]	1.5 (0.02)[a]	4.3 (0.3)[a]
[DTPA^0,D-Tyr^1]octreotide	22.2 (4.5)[a]	27.9 (1.4)[a]	29.6 (1.1)[a]	ND[b]
[DTPA^0,Tyr^3]octreotide	4.7 (0.4)[a]	0.9 (0.03)[a]	4.0 (0.3)[a]	4.8 (0.4)[a]
[DTPA^0,Tyr^3]octreotate	3.2 (0.4)[a]	2.6 (0.4)[a]	0.7 (0.1)[a]	6.3 (0.9)[a]
[DOTA^0,Tyr^3]octreotide	1.6 (0.04)[a]	1.0 (0.02)[a]	1.0 (0.04)[a]	3.6 (0.3)[a]

[a] $P < 0.001$ *versus* control.
[b] ND, not determined.

logues is the use of radiolabeled peptide for radionuclide therapy of receptor-positive lesions. Currently, this application was explored successfully by repeated administration of high doses of [^{111}In-DTPA^0]octreotide in humans (8). However, a β^- particle emitter, such as ^{90}Y, may, in certain cases, appear more suitable for this purpose than the Auger electron emitter ^{111}In. Radiotherapeutic use of ^{90}Y-labeled peptide will lead to a higher and more evenly distributed radiation dose to the tumor because of its larger particle range and tissue penetration. Even tumors with an nonhomogeneous cellular distribution of receptors, such as breast tumors, may respond favorably to treatment with such a ^{90}Y-labeled radiopharmaceutical, whereas treatment with ^{111}In-labeled peptide will not be successful because of the particle range of the Auger electrons, which is only about one cell diameter. Because ^{90}Y-DTPA is unstable, introduction of the DOTA chelator was necessary, enabling stable radiolabeling with ^{90}Y and ^{111}In.

We investigated receptor binding, internalization and biodistribution characteristics of several SS analogues, all labeled with ^{111}In: [DTPA^0]-octreotide, [DTPA^0,Tyr^3]octreotide, [DTPA^0,D-Tyr^1]octreotide, [DOTA^0,Tyr^3]octreotide, and [DTPA^0,Tyr^3]-octreotate. Phe residues were replaced with Tyr to increase the hydrophilicity of the peptides. Furthermore, [DTPA^0,Tyr^3]octreotate, with the C-terminal threonine, was synthesized to investigate the effects of an additional negative charge on clearance and cellular uptake.

For the success of radionuclide therapy, it is important that the radiopharmaceutical is internalized by the tumor cells after binding to the receptor. Internalization of [^{111}In-DTPA^0]octreotide into human neuroendocrine tumor cells was described recently (15). Here, we also observed specific internalization of the tested ^{111}In-labeled peptides. Internalized radioactivity of all radiolabeled peptides was higher than that of [^{111}In-DTPA^0]octreotide, except that of [^{111}In-DTPA^0,D-Tyr^1]octreotide.

The results of the *in vitro* binding studies demonstrated that all unlabeled peptides showed high and specific binding for the octreotide receptors. *In vivo*, uptake of the ^{111}In-labeled peptides in octreotide receptor-expressing tissues was also demonstrated to be highly specific. Our findings further showed that specific uptakes of ^{111}In-labeled [DTPA^0,Tyr^3]octreotide, [DOTA^0,Tyr^3]octreotide, and [DTPA^0,Tyr^3]octreotate in octreotide receptor-expressing tissues were significantly higher than that of [^{111}In-DTPA^0]octreotide at the

Table 4 *Radioactivity in SS receptor-negative organs and blood of rats 24 h after administration of the ^{111}In-labeled peptides*

Tissue radioactivity is expressed as %ID/g [for each group $n \geq 6$, mean (SE)].

Treatment	Blood	Liver	Kidney	Spleen
[DTPA^0]octreotide	0.003 (0.000)	0.05 (0.003)	1.91 (0.11)	0.03 (0.002)
[DTPA^0,D-Tyr^1]octreotide	0.003 (0.001)	0.03 (0.001)[a]	1.52 (0.05)[a]	0.03 (0.002)
[DTPA^0,Tyr^3]octreotide	0.003 (0.001)	0.06 (0.004)	1.39 (0.08)[a]	0.03 (0.002)
[DTPA^0,Tyr^3]octreotate	0.003 (0.000)	0.02 (0.001)[a]	1.96 (0.11)	0.02 (0.001)
[DOTA^0,Tyr^3]octreotide	0.002 (0.001)	0.05 (0.000)	2.32 (0.13)[a]	0.04 (0.001)

[a] $P < 0.01$ *versus* [^{111}In-DTPA^0]octreotide.

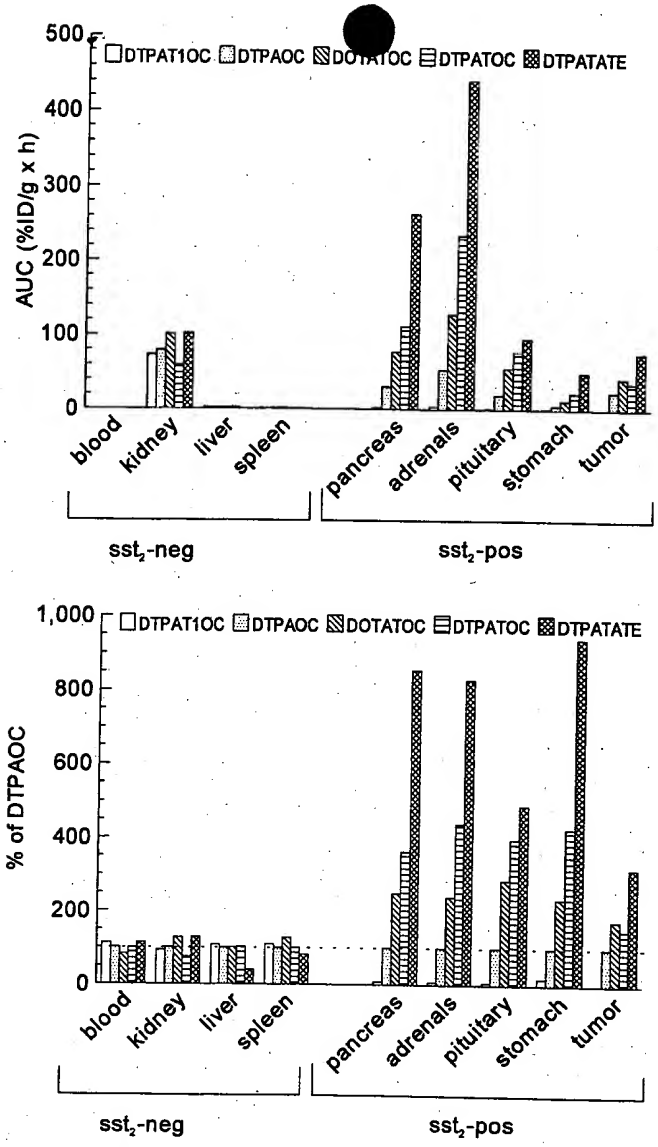

Fig. 3. *Top*, AUC of radioactivity between 4 and 48 h after injection of the different ^{111}In-labeled peptides in rats. *Columns*, mean h·%ID/g. *DTPAOC*, [DTPA^0]octreotide; *DTPAT1OC*, [DTPA^0,D-Tyr^1]octreotide; *DTPAT3OC*, [DTPA^0,Tyr^3]octreotide; *DTPAT3TATE*, [DTPA^0,Tyr^3]octreotate; *DOTAT3OC*, [DOTA^0,Tyr^3]octreotide. *Bottom*, same as *top*, except data represent percentage of [DTPA^0]octreotide AUC (*columns*).

time points tested. Uptake of [^{111}In-DTPA^0,D-Tyr^1]octreotide was significantly lower, in accordance with the lower internalization rate found *in vitro*. In our *in vivo* animal model, [^{111}In-DTPA^0,Tyr^3]octreotate showed the highest uptake in the octreotide receptor-positive organs and tumor of the ^{111}In-labeled peptides tested, also in accordance with the *in vitro* internalization studies. Because blood radioactivity was comparable for all radiolabeled peptides, we also found that [^{111}In-DTPA^0,Tyr^3]octreotate had the highest tumor-to-blood ratio. Uptake of [DTPA^0,Tyr^3]octreotide was higher than that of [DOTA^0,Tyr^3]octreotide, both *in vitro* and *in vivo*, in the octreotide receptor-positive organs; however, uptake in the target, the tumor, was not significantly different for these two radiolabeled peptides; therefore, the therapeutic index of [Tyr^3]octreotide has not been impaired significantly by the replacement of DTPA for DOTA, necessary for ^{90}Y studies (11). We are currently investigating the relationship of the injected peptide mass and uptake in target organs for these two peptides to further elucidate the consequences of the DTPA-to-DOTA replacement.

At radiotherapeutic levels, the high uptake of radioactivity in the octreotide receptor-positive normal organs, such as adrenals and pituitary, should also be considered. We are performing radionuclide therapy studies in normal rats with [DOTA^0,Tyr^3]octreotide radiolabeled with different radionuclides to investigate possible radiotoxic effects on normal organs. However, until now, no radiotoxicity was found in these organs.

The ^{111}In-labeled peptides are rapidly cleared from the body, mostly by the kidneys. However, a significant amount of the dose accumulated in the kidneys, reducing both the scintigraphic sensitivity for detection of small tumors in the perirenal region in the abdomen and the application for radionuclide therapy. It has been reported that renal accumulation of peptides or proteins labeled with radiometals can be reduced by both L- and D-lysine (16–19). Recently, we described that D-lysine administration resulted in a significant reduction of labeled [DTPA^0]octreotide, [DTPA^0,Tyr^3]octreotide, and [DOTA^0,Tyr^3]octreotide uptake in the kidneys without affecting uptake in receptor-positive tissues, which is favorable for both visualization of lesions in the kidney region and for radionuclide therapy, thus bringing these applications further within reach (10).

It can be concluded that ^{111}In-labeled [DTPA^0,Tyr^3]octreotide and, especially, [DTPA^0,Tyr^3]octreotate and their DOTA-coupled counterparts are most promising for scintigraphy and, after coupling to therapeutic radionuclides, for radionuclide therapy of octreotide receptor-positive tumors in humans.

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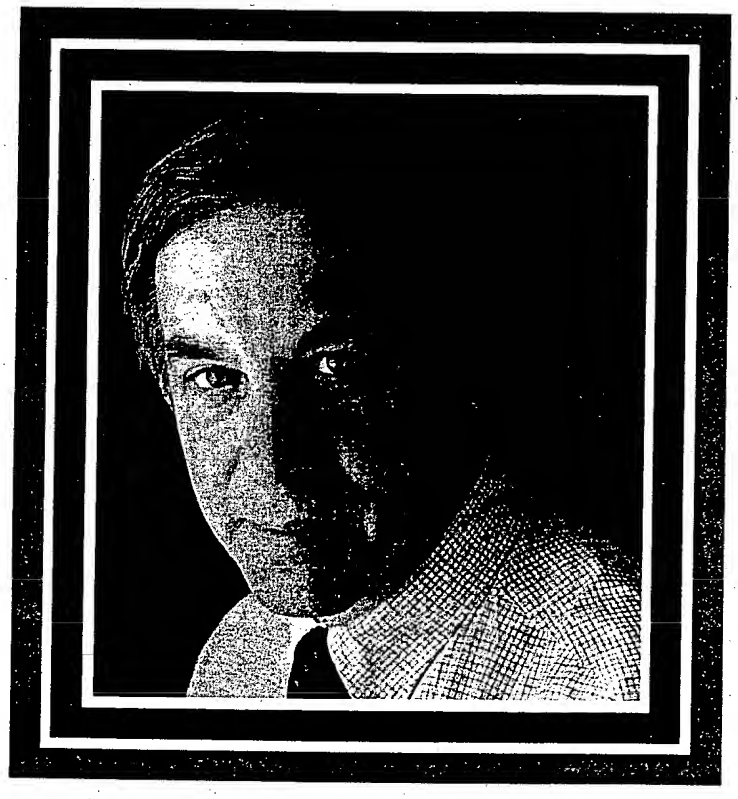

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Articles

Comparison of Four ^{64}Cu-Labeled Somatostatin Analogues in Vitr and in a Tumor-Bearing Rat Model: Evaluation of New Derivatives for Positr n Emission Tomography Imaging and Targeted Radiotherapy[‖]

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Previous studies have shown that modification of the somatostatin analogue octreotide (OC), by substitution of tyrosine for phenylalanine at position 3 and of a C-terminal carboxylic acid for an alcohol, to give Tyr^3-octreotate (Y3-TATE) improved uptake of the peptide in somatostatin receptor-positive tissues. To determine which substitution best accounts for increased target tissue uptake, the peptides containing single modifications, Tyr^3-octreotide (Y3-OC) and octreotate (TATE), were synthesized. These peptides were conjugated to the macrocyclic chelating agent 1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (TETA) and radiolabeled with ^{64}Cu(II). The in vitro receptor binding, in vitro tumor cell uptake, and in vivo distribution properties of ^{64}Cu-labeled TETA-Y3-OC and TETA-TATE were compared to those of [^{64}Cu]TETA-OC and [^{64}Cu]TETA-Y3-TATE. Cu-TETA-TATE (IC_{50} = 0.297 ± 0.0055 nM) and Cu-TETA-Y3-TATE (IC_{50} = 0.308 ± 0.0375 nM) displayed significantly higher binding affinity to somatostatin receptors on CA20948 rat pancreatic tumor membranes than Cu-TETA-Y3-OC (IC_{50} = 0.397 ± 0.0206 nM) and Cu-TETA-OC (IC_{50} = 0.498 ± 0.039 nM). Similarly, the uptakes of [^{64}Cu]TETA-Y3-TATE (60.75 ± 1.21%) and [^{64}Cu]TETA-TATE (55.62 ± 0.16%) into AR42J rat pancreatic tumor cells over a 2-h time period were higher than those of [^{64}Cu]-TETA-Y3-OC (47.20 ± 1.20%) and [^{64}Cu]TETA-OC (34.07 ± 2.24%). The in vitro results suggest that the C-terminal carboxylate may contribute more to enhanced receptor binding and tumor cell uptake than the substitution at the 3-position. Biodistributions in CA20948 tumor-bearing rats showed receptor-mediated uptake of the ^{64}Cu-labeled peptides in somatostatin-rich tissues, including the pituitary, adrenals, pancreas, and tumor. The structure−activity relationships of the four ^{64}Cu-labeled peptides did not show consistent trends in all target tissues, but [^{64}-Cu]TETA-Y3-TATE exhibited tumor uptake 1.75−3.5 times higher than the other derivatives at 4 h postinjection. The greater tumor retention of [^{64}Cu]TETA-Y3-TATE justifies the selection of this agent for future PET imaging and targeted radiotherapy studies.

Introduction

The targeting of somatostatin receptors with radiolabeled peptides has led to the development of agents for both diagnostic imaging and radiotherapy of cancer. Octreotide (OC), an 8-amino acid analogue of somatostatin, has been radiolabeled and used to image somatostatin receptor-positive tumors in humans by positron emission tomography (PET) and single photon emission computed tomography (SPECT). For these purposes, somatostatin analogues have been labeled with a number of β^+- and γ-emitting radionuclides, including ^{111}In, ^{123}I, ^{99m}Tc, ^{68}Ga, ^{64}Cu, ^{18}F, and ^{86}Y.[1−8] In the United States and Europe, ^{111}In-DTPA-OC (In-111 Pentetreotide) is approved for routine clinical use in the diagnosis of neuroendocrine cancer. In addition, widespread interest in targeted radiotherapy has led to the labeling of somatostatin analogues with a variety of cytotoxic radionuclides. For example, [^{161}Tb]DTPA-OC,[9] [^{90}Y]DTPA-OC,[10] [^{188}Re]RC-160,[11] [^{90}Y]DOTA-Tyr^3-OC,[12,13] [^{64}Cu]TETA-OC,[14] and [^{64}Cu]TETA-Tyr^3-TATE[15] are being evaluated for radiotherapeutic efficacy in animal models and clinical trials.

Copper-64 ($t_{1/2}$ = 12.7 h, β^+ = 0.655 MeV (19.3%), β^- = 0.573 MeV (39.6%)) is an attractive radionuclide for both PET imaging and radiotherapy. Large quantities of high-specific activity ^{64}Cu can be produced on demand using a biomedical cyclotron.[16] The applications of ^{64}Cu for PET imaging and targeted radiotherapy through attachment to biologically active molecules have been reviewed.[17] The first ^{64}Cu-labeled somatostatin ana-

[‖] Abbreviations: DTPA, diethylenetriaminepentaacetic acid; DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; TETA, 1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; Y3, tyrosine-3; OC, octreotide; TATE, octreotate; MALDI FTMS, matrix-assisted laser desorption−ionization Fourier transform mass spectrometry.

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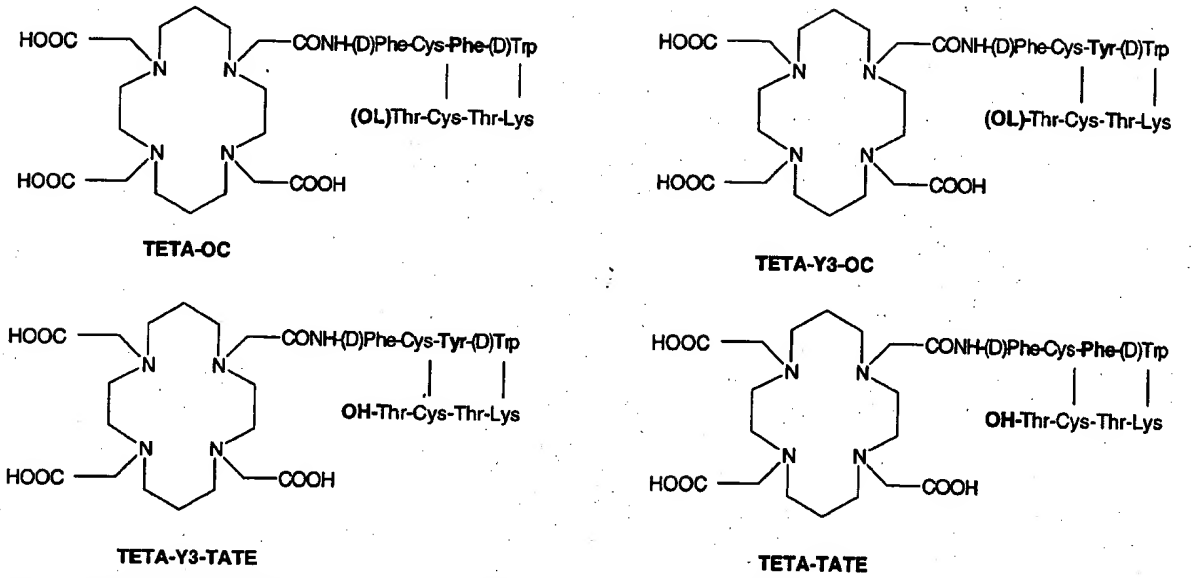

Figure 1. Structures of TETA-OC, TETA-Y3-OC, TETA-Y3-TATE, and TETA-TATE.

logue, [^{64}Cu]TETA-D-Phe^1-octreotide ([^{64}Cu]TETA-OC), displayed high-affinity somatostatin receptor binding in vitro and in vivo.[7] Evaluation of this agent in eight neuroendocrine cancer patients showed that PET imaging with [^{64}Cu]TETA-OC detected more lesions than γ-scintigraphy using [^{111}In]DTPA-OC.[18] Subsequent evaluation of the therapeutic efficacy of [^{64}Cu]TETA-OC has demonstrated growth inhibition of somatostatin receptor-positive tumors in rats at doses exhibiting minimal toxicity.[14]

Studies have shown that subtle modification of the octreotide peptide leads to improved uptake in receptor-rich tissues. The substitutions of a tyrosine (Y) for phenylalanine (F) in the 3-position and of a C-terminal carboxylic acid for an alcohol improved uptake of the peptide in adrenals, pancreas, pituitary, and tumor.[19-21] These findings were confirmed by our own studies, where [^{64}Cu]TETA-D-Phe^1-Tyr^3-octreotate ([^{64}Cu]TETA-Y3-TATE) demonstrated significantly greater uptake than [^{64}Cu]TETA-OC in the somatostatin-rich tissues of two tumor-bearing animal models.[22]

In the current investigation, we examined the effects of single modifications to the octreotide peptide on target tissue uptake, to determine which alteration best accounts for the improvements observed with [^{64}Cu]TETA-Y3-TATE. The substitution of the tyrosine for phenylalanine in the 3-position afforded the peptide Tyr^3-octreotide (Y3-OC), and changing the C-terminus from an alcohol to a carboxylic acid produced the analogue Phe^3-octreotate (TATE). Both of these peptides were subsequently conjugated to TETA (1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) and radiolabeled with ^{64}Cu. We studied receptor-mediated uptake of these two peptides in vitro and in a tumor-bearing animal model and compared the results to those obtained with ^{64}Cu-labeled TETA-OC and TETA-Y3-TATE.

Results

Synthesis and Radiolabeling of Peptides. OC, Y3-OC, TATE, and Y3-TATE (Figure 1) were synthesized by the solid-phase Fmoc method and conjugated with the 1-hydroxybenzotriazole ester of tri-*tert*-butyl TETA on the resin. It should be noted that while these peptides have what is termed TETA conjugated to them, the TETA in use is actually a monoamide derivative of TETA wherein one of the carboxylates has been used to form an amide bond with the peptide. After reversed-phase HPLC, all peptide conjugates were isolated in 90–96% purity. The exact masses of the peptides were confirmed by high-resolution MALDI FTMS, which showed errors of 0.2–6 ppm between observed and calculated values. The ^{64}Cu-labeled peptides were obtained in >98% radiochemical purity, as determined by radio thin-layer chromatography (radio-TLC), in specific activities ranging from 0.5 to 2.5 mCi/μg (18.5–92.5 MBq/μg).

Receptor Binding Assays. The displacement of [^{64}Cu]TETA-OC by the natural copper complexes of TETA-OC, TETA-Y3-TATE, TETA-Y3-OC, and TETA-TATE on rat CA20948 pancreatic tumor cell membranes is shown by the curves presented in Figure 2. All four unlabeled conjugates bound specifically to somatostatin receptors with high affinities. IC_{50} values were 0.308 ± 0.0375 nM for Cu-TETA-Y3-TATE, 0.397 ± 0.0206 nM for Cu-TETA-Y3-OC, and 0.297 ± 0.0055 nM for Cu-TETA-TATE. The value for Cu-TETA-OC, was previously reported to be 0.498 ± 0.039 nM.[14]

AR42J Cell Uptake Studies. The uptakes of ^{64}Cu-labeled TETA-Y3-TATE, TETA-Y3-OC, TETA-OC and TETA-TATE into AR42J rat pancreatic tumor cells during a 2-h incubation at 37 °C are shown in Figure 3. The somatostatin receptor density (B_{max}) on AR42J cells was previously determined by our group to be 148.8 fmol/mg of protein.[23] Thus, under the conditions employed, cell uptake was measured at a 10-fold molar excess of somatostatin receptor to peptide. At 15 min, accumulation of [^{64}Cu]TETA-OC in AR42J cells was 10.23 ± 2.38% of the total activity administered, with uptake increasing to 34.07 ± 2.24% at 2 h. Uptakes of ^{64}Cu-labeled TETA-Y3-OC and TETA-TATE were similar at 15 min (22.77 ± 2.38% and 20.36 ± 1.89%,

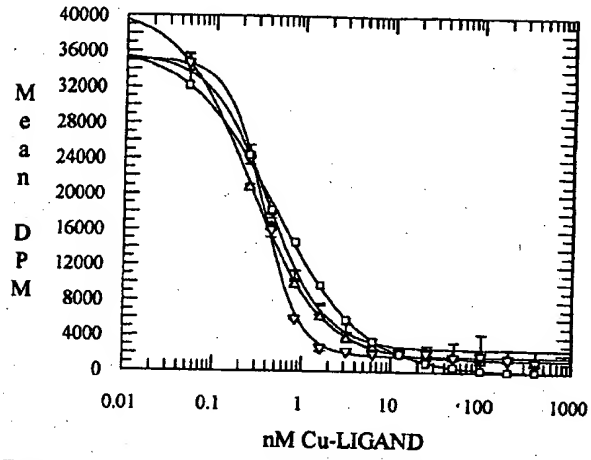

Figure 2. Displacement curves of [^{64}Cu]TETA-OC from CA20948 rat pancreatic tumor cell membranes. Results represent the mean of quadruplicate measurements using ^{nat}Cu-TETA-OC (□), ^{nat}Cu-TETA-Y3-TATE (△), ^{nat}Cu-TETA-Y3-OC (—), or ^{nat}Cu-TETA-TATE (▽).

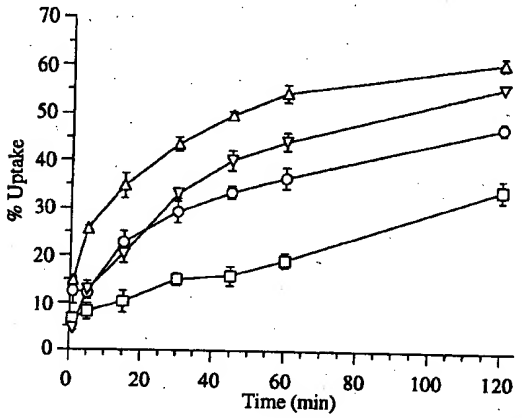

Figure 3. Percentage uptake of [^{64}Cu]TETA-OC (□), [^{64}Cu]-TETA-Y3-TATE (△), [^{64}Cu]TETA-Y3-OC (○), and [^{64}Cu]TETA-TATE (▽) into AR42J cells over time.

respectively) but differed significantly at 2 h (47.20 ± 1.20% and 55.62 ± 0.16%, respectively). Both [^{64}Cu]-TETA-Y3-OC and [^{64}Cu]TETA-TATE exhibited significantly greater accumulation in AR42J cells than [^{64}Cu]-TETA-OC at all time points. Uptake of [^{64}Cu]TETA-Y3-TATE was 34.68 ± 2.53% after 15 min and continued to increase to 60.75 ± 1.21% at 2 h. Over the 2-h experimental period, [^{64}Cu]TETA-Y3-TATE showed the greatest accumulation of the four analogues in AR42J cells. Compared to the other derivatives, the increased uptake of [^{64}Cu]TETA-Y3-TATE was statistically significant at all time points, with the exception of [^{64}Cu]-TETA-TATE at 2 h.

Animal Biodistribution Studies. The uptakes of [^{64}Cu]TETA-Y3-OC and [^{64}Cu]TETA-TATE in pancreas, adrenals, liver, and tumor are shown in Figure 4. For comparison, previously published biodistribution data for [^{64}Cu]TETA-Y3-TATE[22] and [^{64}Cu]TETA-OC[7] are also presented in Figure 4. The results represent biodistributions performed with a similar mass of each radiolabeled peptide (5–8 ng). The results of blocking experiments, using either Y3-TATE or OC to compete with the receptor-mediated uptake of [^{64}Cu]TETA-Y3-TATE, are shown in Figure 5.

Both [^{64}Cu]TETA-Y3-OC and [^{64}Cu]TETA-TATE displayed rapid blood clearance after 1 h. The nontarget organs, e.g., kidney, brain, and liver, showed similar uptake for all four peptide conjugates, with no significant differences. The receptor-rich tissues (adrenals, pancreas, pituitary, and tumor) did not show any significant difference in uptakes between [^{64}Cu]TETA-Y3-OC and [^{64}Cu]TETA-TATE (adrenals, 8.01 ± 1.61% ID/g vs 5.93 ± 1.20% ID/g; pancreas, 4.45 ± 0.96% ID/g vs 5.13 ± 0.92% ID/g; pituitary, 3.41 ± 0.76% ID/g vs 3.69 ± 0.80% ID/g; tumor, 2.17 ± 0.66% ID/g vs 1.76 ± 1.15% ID/g, respectively).

[^{64}Cu]TETA-Y3-TATE had higher uptake in all receptor-rich tissues (except adrenals) than did the other analogues at 1 h (adrenals, 9.07 ± 1.24% ID/g; pancreas, 9.35 ± 1.66% ID/g; pituitary, 6.47 ± 1.77% ID/g; tumor, 2.37 ± 0.44% ID/g) ($p < 0.001$). The trend of adrenal uptakes revealed that ^{64}Cu-labeled TETA-Y3-OC and TETA-Y3-TATE had higher accumulation at 1 and 4 h postinjection than the corresponding Phe^3 analogues. With the exception of the tumor, [^{64}Cu]TETA-Y3-TATE, [^{64}Cu]TETA-Y3-OC, and [^{64}Cu]TETA-TATE all demostrated at least 2-fold higher uptake than [^{64}Cu]TETA-OC in receptor-positive organs. At 1 h, tumor uptakes of [^{64}Cu]TETA-Y3-OC and [^{64}Cu]TETA-TATE were similar to the values obtained with [^{64}Cu]TETA-Y3-TATE and [^{64}Cu]TETA-OC. However, at 4 h, the tumor uptake of [^{64}Cu]TETA-Y3-TATE (2.22 ± 0.26% ID/g) was significantly higher than that of [^{64}Cu]TETA-Y3-OC (1.28 ± 0.25% ID/g) and [^{64}Cu]TETA-TATE (0.63 ± 0.52% ID/g), as well as the tumor uptake of [^{64}Cu]TETA-OC at 3 h (0.63 ± 0.05% ID/g).

In the ligand competition experiments, more than 90% of the uptake of [^{64}Cu]TETA-Y3-TATE in somatostatin-rich tissues was blocked with a co-injection of either unlabeled Y3-TATE or unlabeled OC. At 1 h, co-injection of Y3-TATE decreased the pancreatic uptake of [^{64}Cu]TETA-Y3-TATE significantly more than co-injection of OC (0.15 ± 0.02% ID/g vs 0.76 ± 0.13% ID/g, respectively) ($p < 0.005$). The same trend is seen in the adrenals (0.17 ± 0.02% ID/g for Y3-TATE and 0.26 ± 0.09% ID/g for OC) and the tumor (0.22 ± 0.02% ID/g for Y3-TATE and 0.64 ± 0.10% ID/g for OC) at 1 h postinjection. Interestingly, the bone also shows receptor-mediated uptake of [^{64}Cu]TETA-Y3-TATE. Using Y3-TATE as the blocking agent, bone uptake was decreased from 0.61 ± 0.08% ID/g to 0.09 ± 0.02% ID/g at 1 h; a blocking dose of OC decreased the bone uptake to 0.13 ± 0.02% ID/g at the same time point. Co-injection with blocking peptides did not have a significant effect on uptake in nontarget organs.

Discussion

[^{64}Cu]TETA-OC is currently being investigated for clinical PET imaging of neuroendocrine cancer.[18] Preliminary results with this compound are encouraging in that more tumors have been visualized with this agent than with [^{111}In]DTPA-OC. [^{64}Cu]TETA-OC has also been evaluated for targeted radiotherapy in a tumor-bearing rat model.[14] However, it suffers from the disadvantages of less than optimal blood clearance and rapid tumor clearance. On the basis of previous results obtained with ^{111}In-labeled octreotide analogues,[20,21] we have evaluated [^{64}Cu]TETA-Y3-TATE in vitro and in

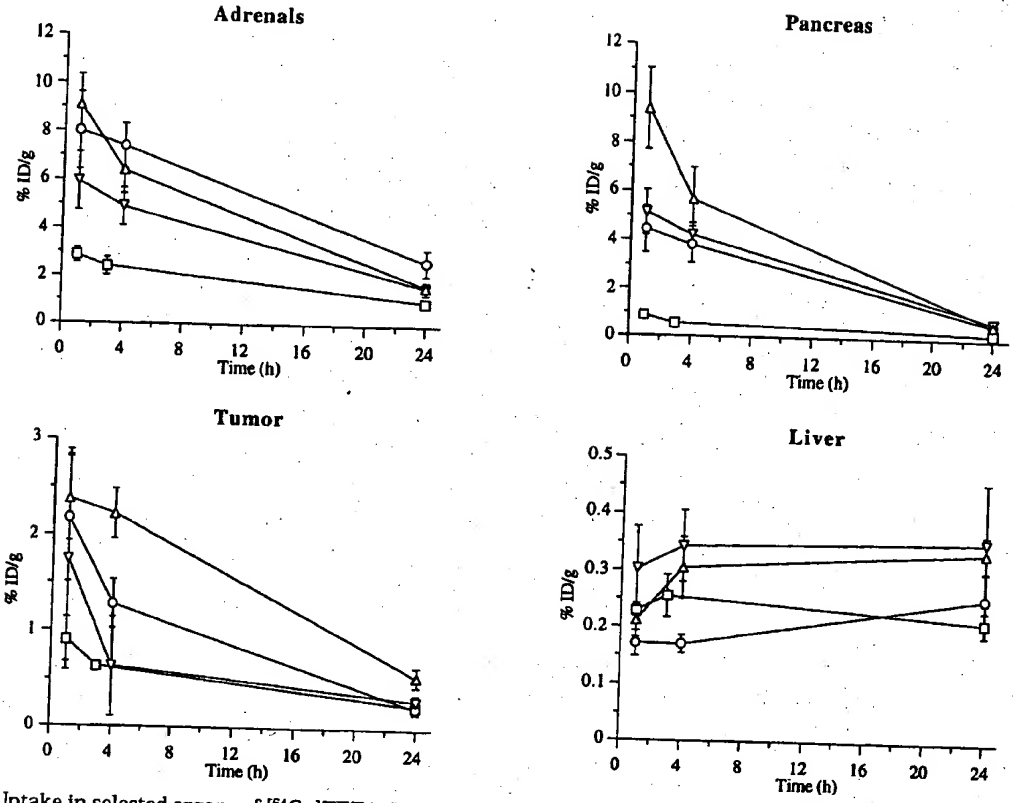

Figure 4. Uptake in selected organs of [^{64}Cu]TETA-OC (□), [^{64}Cu]TETA-Y3-TATE (△), [^{64}Cu]TETA-Y3-OC (○), and [^{64}Cu]TETA-TATE (▽) in Lewis rats bearing CA20948 rat pancreatic tumors. Standard deviations (SD) are indicated; all data were corrected for radiodecay. Note differences in *y*-axis scales.

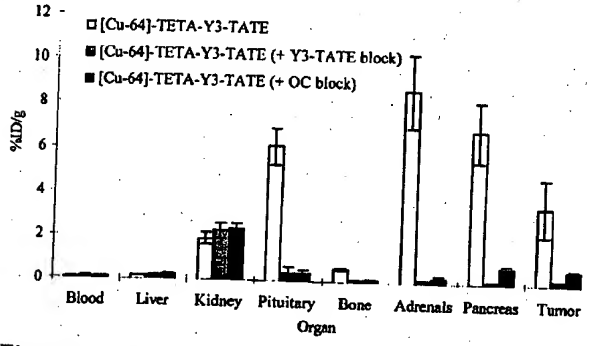

Figure 5. Biodistributions at 1 h of [^{64}Cu]TETA-Y3-TATE, [^{64}Cu]TETA-Y3-TATE co-injected with 150 μg of Y3-TATE, and [^{64}Cu]TETA-Y3-TATE co-injected with 150 μg of OC in Lewis rats bearing CA20948 rat pancreatic tumors. Standard deviations (SD) are indicated; all data were corrected for radiodecay.

two animal models as a potential agent for PET imaging[22] and targeted radiotherapy.[15] [^{64}Cu]TETA-Y3-TATE demonstrated rapid blood clearance in CA20948-bearing Lewis rats, with tumor uptake twice that of [^{64}Cu]TETA-OC. Moreover, tumor:blood ratios were over 4-fold higher at 1 h for [^{64}Cu]TETA-Y3-TATE.

[^{64}Cu]TETA-Y3-TATE differs from the parent compound, [^{64}Cu]TETA-OC, by the substitutions of tyrosine for phenylalanine in the 3-position and a C-terminal carboxylic acid for an alcohol. The current study was undertaken to determine how these modifications contribute to the increase in uptake of [^{64}Cu]TETA-Y3-TATE in receptor-rich tissues. Two peptides, TETA-TATE and TETA-Y3-OC, were synthesized, radiolabeled with ^{64}Cu, and evaluated in Lewis rats bearing CA20948 pancreatic tumors. Compared to the parent peptide OC, TETA-Y3-OC contains the substitution of tyrosine in the 3-position, while TETA-TATE incorporates the change in C-terminus from an alcohol to an acid.

In vitro receptor binding studies showed that all peptides evaluated bound specifically to somatostatin receptors on CA20948 membranes with high relative affinities. The parent compound, Cu-TETA-OC, had the lowest affintity for the receptor, while Cu-TETA-Y3-TATE and Cu-TETA-TATE had the highest affinities. Cu-TETA-Y3-OC exhibited a lower affinity for the receptor than the TATE derivatives, but its IC_{50} value was still significantly lower than that of Cu-TETA-OC. These results suggest that the C-terminal modification may contribute more to high-affinity receptor binding than the substitution at position 3.

The AR42J rat pancreatic carcinoma cell line is also known to express somatostatin receptors both in vitro and in vivo.[24,25] To evaluate and compare the cellular uptake of the radiolabeled peptides in vitro, the AR42J cell line was utilized. Under the conditions employed, the mass of each peptide added was identical, and the somatostatin receptor concentration was 10-fold greater than the peptide concentration. Therefore the results obtained are a direct comparison of the accumulation rates of the analogues and likely represent a combination of membrane binding, internalization, and cellular retention of the compounds. The data revealed that [^{64}Cu]TETA-Y3-TATE had the highest uptake in AR42J cells, followed by [^{64}Cu]TETA-TATE, [^{64}Cu]TETA-Y3-

OC, and [^{64}Cu]TETA-OC in descending order. As in the case of the receptor binding studies, these results showed that the C-terminal carboxyl modification makes a greater contribution to increased cell uptake than the substitution at position 3.

The results of the cell uptake studies are in agreement with the findings of de Jong et al.,[20,26] who reported that the amounts of ^{111}In-labeled somatostatin analogues internalized into CA20948 and AR42J cells followed the trend DTPA-Y3-TATE > DTPA-Y3-OC > DOTA-Y3-OC > DTPA-OC. While those studies distinguished between internalized and membrane-bound ligand, they did not include DTPA- or DOTA-TATE derivatives, so the individual contributions of the C-terminal and 3-position modifications could not be assessed. The studies by de Jong et al. were conducted at peptide concentrations as low as 100 pM. The results described here were obtained at peptide concentrations of exactly 30 pM to give a receptor:ligand molar ratio of 10:1. Receptor excess is desirable for comparison of cellular uptakes because it mimics the physiological conditions of tumor targeting in vivo.

The rat biodistribution studies clearly demonstrated that the uptakes of [^{64}Cu]TETA-Y3-TATE, [^{64}Cu]TETA-Y3-OC, and [^{64}Cu]TETA-TATE in receptor-positive normal tissues are significantly higher at 1 and 4 h than that of [^{64}Cu]TETA-OC. The tyrosine-substituted analogues, [^{64}Cu]TETA-Y3-TATE and [^{64}Cu]TETA-Y3-OC, showed higher uptake in the adrenals than the corresponding phenylalanine-substituted derivatives. This finding suggests that the presence of the tyrosine residue may be responsible for increased adrenal uptake, possibly a result of the increased hydrophilicities of these peptides. In the pancreas and pituitary, [^{64}Cu]-TETA-Y3-TATE showed the highest uptakes at 1 h, while [^{64}Cu]TETA-TATE and [^{64}Cu]TETA-Y3-OC had similar intermediate uptakes, and [^{64}Cu]TETA-OC exhibited much lower uptakes than the other three analogues. In these target tissues, the combination of C-terminal and residue 3 modifications may have a synergistic effect on uptake. These observations are consistent with the findings of de Jong et al.,[20,21] who showed increased target tissue uptake with ^{111}In-labeled DTPA-Y3-OC and DTPA-Y3-TATE derivatives.

The in vivo ligand competition experiments demonstrated that uptake of [^{64}Cu]TETA-Y3-TATE is receptor-mediated in all target tissues. Moreover, Y3-TATE was generally more effective as a blocking agent than OC, a finding which may be attributable to its higher affinity for somatostatin receptors or differences in internalization rates or uptake kinetics. The same ligand competition effect was also observed in bone, suggesting that bone uptake of [^{64}Cu]TETA-Y3-TATE was also receptor-mediated.

Tumor uptakes of the four ^{64}Cu-labeled octreotide analogues at 1 h were more similar than the uptakes in other target tissues. At this time point, [^{64}Cu]TETA-OC had the lowest tumor uptake. While [^{64}Cu]TETA-Y3-TATE had the highest accumulation in tumor at 1 h, this value was not significantly different than those obtained with ^{64}Cu-labeled TETA-TATE and TETA-Y3-OC. However, at 4 h postinjection, tumor uptake of [^{64}Cu]TETA-Y3-TATE was 1.75−3.5 times higher than those of the other analogues. The longer residence time of [^{64}Cu]TETA-Y3-TATE in the tumor may increase its efficacy for targeted radiotherapy and justify future therapy studies using this agent.

It is evident from these investigations that modification of the 3-position amino acid and alteration of the C-terminus both contribute to increased target tissue uptake of ^{64}Cu-labeled octreotide analogues. While the structure−activity relationships of these four analogues do not show consistent uptake trends in all target tissues that identify the superior compound, the greater accumulation and retention of [^{64}Cu]TETA-Y3-TATE in tumor provide a rationale to select this agent for future targeted radiotherapy studies. We are continuing to evaluate the therapeutic efficacy of [^{64}Cu]TETA-Y3-TATE in the CA20948 rat model in preparation for clinical trials.

Experimental Section

Materials. ^{64}Cu was produced on a biomedical cyclotron at Washington University School of Medicine by previously reported methods.[16] All chemicals, unless otherwise stated, were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). All solutions were prepared using ultrapure water (18 MΩ-cm resistivity). Thin-layer chromatography was performed using Whatman MKC$_{18}$F reversed-phase TLC plates with 10% ammonium acetate:methanol (30:70) as the mobile phase. Radio-TLC detection was accomplished using a BIOSCAN System 200 imaging scanner (Washington, DC). Radioactive samples were counted on a Beckman 8000 γ counter (Irvine, CA). Adult male Lewis rats (230−290 g) were purchased from Harlan Sprague−Dawley, Inc. (Indianapolis, IN). The rat pancreatic tumor CA20948[27] was obtained from the Tumor Bank at Biomeasure, Inc. (Hopkinton, MA) and was maintained by serial passage in animals.

Peptide Synthesis. Solid-phase peptide synthesis (SPPS) was performed on an Applied Biosystems model 432A "synergy" peptide synthesizer employing the Fmoc (9-fluorenylmethoxycarbonyl) method. Instrument protocol required 25 μmol of subsequent Fmoc-protected amino acids activated by a combination of 1-hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The Fmoc-protected amino acids were purchased commercially unless otherwise stated; the prepacked amino acids were obtained from Perkin-Elmer (Norwalk, CT), while those unavailable in prepacked form, such as the D-amino acids and Fmoc-Cys(Acm), were supplied by BACHEM Bioscience, Inc. (King of Prussia, PA) or Novabiochem (San Diego, CA). Tri-*tert*-butyl TETA was synthesized internally by a modification of the published procedure.[28] Exact mass measurements on the peptide conjugates were performed by Mass Consortium (San Diego, CA), using an IonSpec Fourier transform ion cyclotron mass spectrometer with a 4.7-T superconducting magnet. Samples in 2,5-dihydroxybenzoic acid matrix were irradiated with a nitrogen laser (LaserScience, Inc.) operated at 337 nm.

The synthesis of TETA-Y3-TATE, TETA-OC, TETA-Y3-OC, and TETA-TATE was accomplished by previously reported methods.[22] The peptide conjugates were purified by reversed-phase HPLC, using a Vydac Protein & Peptide C$_{18}$ column (2.2 × 25 cm) and a linear gradient from 10% to 70% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH_3CN) over 40 min at a flow rate of 10 mL/min. Detection was accomplished at 230 nm. Pure fractions were identified by analytical HPLC using two diverse systems: system A, HPLC on a Vydac diphenyl (219TP54) column (0.46 × 25 cm) and a linear gradient from 2% to 98% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/CH_3CN) over 100 min at a flow rate of 1 mL/min, with detection at 214 and 280 nm; system B, reversed-phase HPLC on a Vydac Protein & Peptide C$_{18}$ column (0.46 × 25 cm), with detection at 214 nm. For TETA-Y3-TATE, TETA-Y3-OC, and TETA-TATE, analytical reversed-phase HPLC was performed using a solvent gradient starting with

0% solvent B for 2 min, followed by a linear gradient from 0% to 70% solvent B (solvent A, 0.1% TFA/5% CH_3CN; solvent B, 0.1% TFA/90% CH_3CN) over 15 min at a flow rate of 0.5 mL/min. For TETA-OC, analytical reversed-phase HPLC was carried out using a linear gradient from 5% to 70% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH_3CN) over 15 min at a flow rate of 2 mL/min. The peptides were also analyzed by high-resolution MALDI FTMS. TETA-Y3-TATE: HPLC retention times = 33.0 min (system A), 11.2 min (system B); MALDI FTMS *m/z* calcd for $C_{67}H_{95}N_{14}O_{19}S_2$ $(M + H)^+$ = 1463.6339, found 1463.6343. TETA-OC: HPLC retention times = 35.9 min (system A), 10.8 min (system B); MALDI FTMS *m/z* calcd for $C_{67}H_{97}N_{14}O_{17}S_2$ $(M + H)^+$ = 1433.6598, found 1433.6609. TETA-Y3-OC: HPLC retention times = 32.5 min (system A), 11.1 min (system B); MALDI FTMS *m/z* calcd for $C_{67}H_{97}N_{14}O_{18}S_2$ $(M + H)^+$ = 1449.6547, found 1449.6646. TETA-TATE: HPLC retention times = 36.3 min (system A), 12.3 min (system B); MALDI FTMS *m/z* calcd for $C_{67}H_{95}N_{14}O_{18}S_2$ $(M + H)^+$ = 1447.6390, found 1447.6404.

Radiolabeling of Peptide Conjugates. The conjugated peptides were labeled with ^{64}Cu(II) according to previously reported methods for the preparation of [^{64}Cu]TETA-OC[7] and [^{64}Cu]TETA-Y3-TATE.[22] Briefly, 1−5 mCi (37−185 MBq) of ^{64}Cu in 0.1 M ammonium acetate, pH 5.5, was added to 1−10 μg of the peptide conjugate in 0.1 M ammonium acetate, pH 5.5. Gentisic acid (1 mg/mL) was added to the labeling mixture to counteract the effects of radiolysis. The solution was incubated for 1 h at room temperature. The radiolabeled peptide was purified on a C-18 SepPak cartridge, using 100% ethanol as the elution solvent, and radiochemical purity was determined by radio-TLC.

Receptor Binding Assays. The receptor binding assays were performed using [^{64}Cu]TETA-OC on membranes obtained from CA20948 tumors harvested from euthanized rats. The competing ligands, ^{nat}Cu-TETA-OC, ^{nat}Cu-TETA-Y3-TATE, ^{nat}Cu-TETA-Y3-OC, and ^{nat}Cu-TETA-TATE, were prepared by the reaction of high-purity natural copper acetate, using the same procedure described above for preparation of the ^{64}Cu-labeled peptides. Purity of the final products were confirmed by HPLC, using the same method described for purification of the TETA conjugates. IC_{50} values were determined according to previously published methods,[14] using the Millipore MultiScreen assay system (Bedford, MA). Data analysis was performed using the programs GraFit (Erithacus Software, U.K.), LIGAND (NIH, Bethesda, MD), and GraphPad PRISM (San Diego, CA). Each data point represents the mean of four experimental values.

Cell Uptake Studies. The apparatus and procedures for the cell uptake experiments are based on previously described methods.[29,30] Briefly, the AR42J cell line was maintained by serial passage in monolayers in Dulbecco's modified Eagle's media (DMEM), supplemented with 10% fetal bovine serum, in a humidified 5% CO_2 atmosphere at 37 °C. Viability of the cells and cell numbers were measured by trypan blue exclusion procedures using a hemacytometer. The cell viability before and after the experiments was determined to be >95% in all cases. Cells were harvested from monolayers with cell dissociation solution (Sigma Chemical Co., St. Louis, MO) and resuspended in fresh DMEM media at a concentration of 2 × 10^6 cells/mL. An aliqout of 0.3 pmol of the radiolabeled peptide (1.11 μCi of [^{64}Cu]TETA-OC, 2.13 μCi of [^{64}Cu]TETA-Y3-TATE, 1.94 μCi of [^{64}Cu]TETA-Y3-OC, or 1.93 μCi of [^{64}Cu]TETA-TATE) was added to 10 mL of cells, which were incubated at 37 °C with continuous agitation. At 1, 5, 15, 30, 45, 60, and 120 min triplicate 200-μL aliquots were removed and placed in ice. The cells were immediately isolated by centrifugation, and the percent uptake of the compound into the cells was calculated as described.[30]

Animal Biodistribution Studies. Using a 21G Trocar, the somatostatin receptor-positive rat pancreatic tumor CA20948 (1-mm^3 piece) was implanted subcutaneously into the nape of the neck of male Lewis rats (230−290 g). The tumors were allowed to grow for 10 days, until approximately 4 g in size. The ^{64}Cu-labeled peptide conjugate (5.4 μCi, 5 ng) was injected intravenously via the tail vein into CA20948 tumor-bearing Lewis rats. Animals were euthanized at 1, 4, and 24 h postinjection. The tumor, blood, lung, liver, spleen, kidney, muscle, fat, heart, brain, pituitary, bone, adrenals, pancreas, stomach, small intestine, upper large intestine, and lower large intestine were removed, drained of blood, weighed, and counted in a γ counter. By comparison with a standard representing the injected dose per animal, the samples were corrected for radioactive decay, to calculate percent injected dose per gram (% ID/g) of tissue and percent injected dose per organ (% ID/organ).

Ligand Competition Experiments. [^{64}Cu]TETA-Y3-TATE (5.4 μCi, 5 ng) was injected intravenously via the tail vein into CA20948-bearing Lewis rats. Two additional groups of animals were co-injected with [^{64}Cu]TETA-Y3-TATE (5.4 μCi, 5 ng) and either 150 μg of unlabeled Y3-TATE or 150 μg of unlabeled OC. All three groups of animals were sacrificed at 1 h postinjection, after which biodistributions were obtained as described above.

Statistical Methods. To compare differences between the ^{64}Cu-labeled peptides, a Student's *t*-test was performed. Differences at the 95% confidence level ($p < 0.05$) were considered significant.

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Supporting Information Available: Tables of mean percent injected dose per gram (% ID/g) of [^{64}Cu]TETA-Y3-OC, [^{64}Cu]TETA-TATE, and [^{64}Cu]TETA-Y3-TATE with two blocking agents and percent injected dose per organ (% ID/organ) with standard deviations for 13 tissues and 3 time points evaluated and also synthesis of mono-*N*-(carboxymethyl)-tris-*N,N,N*-(*tert*-butyloxycarbonylmethyl)cyclam (tri-*tert*-butyl TETA) from cyclam. This information is available free of charge via the Internet at http://pubs.acs.org.

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Radiotherapy and Dosimetry of ^{64}Cu-TETA-Tyr^3-Octreotate in a Somatostatin Receptor-positive, Tumor-bearing Rat Model[1]

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ABSTRACT

^{64}Cu [$T_{1/2}$= 12.8 h; β^+ = 0.655 MeV (19%); β^- = 0.573 MeV (40%)] has shown promise as a radioisotope for targeted radiotherapy. It has been demonstrated previously that the somatostatin analogue ^{64}Cu-TETA-octreotide (^{64}Cu-TETA-OC, where TETA is 1,4,8,11-tetraazacyclotetradecane-*N,N′,N″,N‴*-tetraacetic acid) significantly inhibited the growth of somatostatin receptor-positive CA20948 rat pancreatic tumors in Lewis rats (C. J. Anderson *et al.*, J. Nucl. Med., *39:* 1944–1951, 1998). In this study, we evaluated the radiotherapeutic efficacy of a new ^{64}Cu-labeled somatostatin analogue, ^{64}Cu-TETA-Tyr^3-octreotate (^{64}Cu-TETA-Y3-TATE), in CA20948 tumor-bearing rats. A single dose of 15 mCi (555 MBq) of ^{64}Cu-TETA-Y3-TATE was shown to be more effective in reducing tumor burden than the same dose of ^{64}Cu-TETA-OC. In multiple dose experiments, tumor-bearing rats were administered three doses of either 10 or 20 mCi (370 or 740 MBq) of ^{64}Cu-TETA-Y3-TATE at 48-h intervals. Rats given 3 × 10 mCi (3 × 370 MBq) showed extended mean survival times compared with rats given a single dose; however, no complete regressions occurred. Complete regression of tumors was observed for all rats treated with 3 × 20 mCi (3 × 740 MBq), with no palpable tumors for ~10 days; moreover, the mean survival time of these rats was nearly twice that of controls. Toxicity was determined by physical appearance and hematological and enzyme analysis, which revealed no overt toxicity and only transient changes in blood and liver chemistry. Absorbed dose estimates showed the dose-limiting organ to be the kidneys. The radiotherapy results, along with absorbed dose estimates to target and clearance organs, confirm that ^{64}Cu-labeled somatostatin analogues warrant continued consideration as agents for targeted radiotherapy.

INTRODUCTION

Somatostatin analogues have been investigated for utility in scintigraphic and PET[3] imaging of cancer in humans. For example, ^{111}In-pentetreotide (^{111}In-DTPA-octreotide; Refs. 1 and 2) has been approved for routine clinical use in the diagnosis of neuroendocrine cancer in the United States and Europe. Somatostatin analogues have also been labeled with other radionuclides and evaluated as possible radiotherapeutic agents. Targeted radiotherapy studies have been performed in animal models with somatostatin analogues labeled with ^{90}Y (3–5), ^{111}In (6), and ^{64}Cu (7) with varying degrees of success, and of these agents, ^{111}In-DTPA-octreotide (8–10) and ^{90}Y-DOTA-Tyr^3-octreotide (^{90}Y-SMT 487, ^{90}Y-DOTATOC, or ^{90}Y-DOTA-Y3-OC; Ref. 11) are being investigated in ongoing clinical radiotherapy trials.

Improvement in target tissue uptake of radiolabeled somatostatin analogues has been the focus of a number of studies. It has been shown that substitution of a tyrosine (Y) for phenylalanine (F) in the 3-position and changing the C-terminus from an alcohol to a carboxylic acid increases uptake of the peptide in receptor-rich tissues (12–14). This has been confirmed by our own studies, where ^{64}Cu-TETA-Tyr^3-octreotate (^{64}Cu-TETA-Y3-TATE; Fig. 1) demonstrated significantly greater uptake in somatostatin-rich tissues in two tumor-bearing animal models (Lewis rats bearing CA20948 tumors and severe combined immunodeficient mice bearing AR42J tumors) compared with ^{64}Cu-TETA-octreotide (^{64}Cu-TETA-OC; Fig. 1; Refs. 15 and 16).

^{64}Cu [$T_{1/2}$ = 12.8 h; β^+ = 0.655 MeV (19%); β^- = 0.573 MeV (40%)] has diverse applications in radiopharmaceutical chemistry for PET imaging (17) as well as therapy (7, 18). Moreover, ^{64}Cu can be produced on demand in high yield and in high specific activity on a small biomedical cyclotron (19), making it a radionuclide available to many medical institutions.

We report herein an investigation into the radiotherapeutic potential of ^{64}Cu-TETA-Y3-TATE in CA20948 tumor-bearing

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[3] The abbreviations used are: PET, positron emission tomography; Y3, tyrosine-3; TETA, 1,4,8,11-tetraazacyclotetradecane-*N,N′,N″,N‴*-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; DOTA, 1,4,7,10-tetraazacyclododecane- *N,N′,N″,N‴*-tetraacetic acid; OC, octreotide; TATE, octreotate; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ID/g, injected dose per gram; MIRD, medical internal radiation dose; ROI, region of interest.

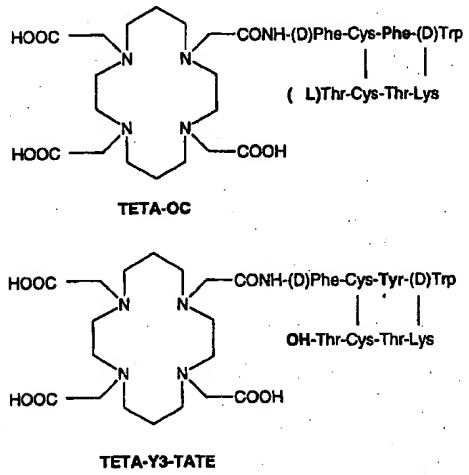

Fig. 1 Structures of TETA-OC (*top*) and TETA-Y3-TATE (*bottom*).

rats, a model of somatostatin receptor-positive pancreatic cancer. The therapeutic efficacy of ^{64}Cu-TETA-Y3-TATE was compared with ^{64}Cu-TETA-OC and control agents. In addition, hematological, liver, and kidney assays were performed to evaluate the potential toxicity of the ^{64}Cu-TETA-Y3-TATE. Human absorbed dose estimates for ^{64}Cu-TETA-Y3-TATE were calculated from both rat biodistribution data and PET imaging of a baboon.

MATERIALS AND METHODS

Synthesis of ^{64}Cu-TETA-OC and ^{64}Cu-TETA-Y3-TATE. ^{64}Cu was produced on a biomedical cyclotron at the Washington University School of Medicine by methods reported previously (19). ^{64}Cu-TETA-OC and ^{64}Cu-TETA-Y3-TATE were prepared according to literature methods (15, 16, 20), in specific activities ranging from 1.25 to 2.5 mCi/μg (46–93 MBq/μg).

Animal Models. All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by Washington University's Animal Studies Committee. The rat pancreatic tumor CA20948 (21) was obtained from the Tumor Bank at Biomeasure, Inc. (Hopkinton, MA). Adult male Lewis rats (230–290 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The CA20948 cell line was maintained by serial passage in animals. In rat experiments, male Lewis rats were injected with a 1-mm^3 tumor section of CA20948 tumor 10 days prior to treatment as described previously (7).

Radiotherapy Experiments (Single Dose). Tumor-bearing rats (tumor volume, 0.3–1.0 cm^3) were injected with one dose of 200 μl of 0.1 M NH_4OAc (buffer), 15 μg of TETA-Y3-TATE, 15.5 mCi (574 MBq) of ^{64}Cu-TETA-OC, or 15.5 mCi (574 MBq) of ^{64}Cu-TETA-Y3-TATE. The tumor volume was measured every 1–3 days (using calipers), and rats were sacrificed by administration of Metofane, followed by cervical dislocation, when the tumors reached a volume of ~10 cm^3 or became ulcerated.

Radiotherapy Experiments (Multiple Dose). Two different multiple dose protocol experiments were carried out. In the first experiment, one group of tumor-bearing rats (tumor volume, 0.3–1.5 cm^3) received three 10-mCi (370 MBq) doses of ^{64}Cu-TETA-Y3-TATE, with a control group receiving equivalent masses of unlabeled TETA-Y3-TATE. Rats were injected 10, 12, and 14 days after implantation of tumor cells. The second experiment was identical, except that the treated group of rats received three 20-mCi (740 MBq) doses of ^{64}Cu-TETA-Y3-TATE 10, 12, and 14 days after implantation, and the control group received equivalent masses of unlabeled TETA-Y3-TATE on those days. Tumor volumes were measured, and animals were sacrificed in a manner identical to that described for the single-dose radiotherapy experiments.

Blood Chemistry. Hematological, liver, and kidney chemistries were studied for the group of tumor-bearing rats that received 3 × 20 mCi. These results were compared with those obtained from rats treated with control agents. In each group, anesthetized rats were weighed, and blood was removed by cardiac puncture during the posttherapy survival period. Toxicity analysis was performed by the Diagnostic Services Laboratory in the Department of Comparative Medicine at Washington University School of Medicine. The hematology analysis included WBC counts, RBC counts, platelet counts, as well as measurement of hemoglobin, hematocrit, and differential WBCs. Liver and kidney analysis included blood urea nitrogen, creatinine, ALP, ALT, and AST.

Effect of Specific Activity on Biodistribution of Radiolabeled Peptide. To determine the effect of peptide mass on the biodistribution of ^{64}Cu-TETA-Y3-TATE, groups of CA20948 tumor-bearing rats (n = 5) were coinjected with 10 ng of ^{64}Cu-TETA-Y3-TATE (5 μCi, 0.2 MBq) and a known mass of TETA-Y3-TATE to give final injectates of 10, 50, 100, 500, 1000, and 5000 ng. All animals were sacrificed at 1 h, and % ID/g and % ID/organ values of selected tissues and organs were determined.

Rat Dosimetry. The estimated human absorbed doses of ^{64}Cu-TETA-Y3-TATE to normal organs were obtained using biodistribution data in CA20948 tumor-bearing rats, according to methods described previously (7). ^{64}Cu-TETA-Y3-TATE (35 μCi, 1.3 MBq) was injected i.v., and the rats were euthanized by Metofane overdose and cervical dislocation at 1, 3, 6, 12, 24, 36, and 48 h after injection. The rats for the 48-h time point were housed in metabolism cages to determine % ID excreted in urine and feces at 1, 3, 6, 17, 24, 43, and 48 h. Time-activity curves were generated for 12 organs. Cumulative activity (μCi-h or kBq-h) was determined by numerically integrating the area under the time-activity curves. Human dose estimates were then calculated using standard MIRD techniques, and S-values (mean absorbed dose per unit cumulative activity) for ^{64}Cu were obtained from the MIRDOSE3 program (22). Bone activity was assumed to be distributed equally between the trabecular bone and cortical bone. The absorbed dose to the CA20948 rat tumor was determined from biodistribution data using methods described previously (18). Briefly, time-activity data were determined by combining the tumor data from different time points after injection. Each tumor was excised and weighed, and an S-value was calculated for the average tumor size for each time point, assuming a spherical tumor of unit density. Nonpenetrat-

ing emissions of ^{64}Cu were assumed to be completely absorbed in the tumor. For penetrating emissions, appropriate specific absorbed fractions for the 511 and 1340 keV photons of ^{64}Cu were used. This approach assumes that tumors of similar size in different animals demonstrate similar uptake characteristics, and the resulting absorbed dose is an average of that absorbed by each tumor.

Baboon Dosimetry. The biodistribution of ^{64}Cu-TETA-Y3-TATE was also determined in a 25-kg male baboon by PET imaging. PET imaging was performed using a Siemens/CTI ECAT EXACT PET system (CTI PET Systems, Knoxville, TN) to determine the biodistribution of 4.6 mCi (170.2 MBq) of ^{64}Cu-TETA-Y3-TATE over the first 18 h after injection. Images of the animal's torso were acquired at 30-min intervals from 0 to 3 h and then again at 18 h after injection. The baboon was anesthetized for the first 3 h and then repositioned to approximately the same position the following day.

Activity concentration values were derived from the PET images, which were calibrated previously against the same dose calibrator (Capintec, Ramsey, NJ) used to assay the injected dose. Corrections for photon attenuation, random coincidences, deadtime, and scatter were applied. Images were reconstructed with filtered backprojection and modest smoothing (Hann, cut-off 0.3 pixels^{-1}), so that most organs could be clearly identified. ROIs were drawn over liver, spleen, kidneys, bladder, blood pool, red marrow, and muscular soft tissue and were used to estimate total organ accumulations of the compound. Blood activity was taken to be the average of the maximum pixels in five to six adjacent slices through the left ventricle, which was necessary to avoid partial volume effects in the moving heart. Liver activity was taken as the average value in a large ROI centered in the liver and averaged > five to six slices. Kidney activity, although trapped primarily in the renal cortex, was taken as the average value inside a ROI outlining the entire kidney and was assumed to be uniformly distributed in the organ. ROI values were decay-corrected to the time of injection, extrapolated where necessary by standard human organ and blood volumes, and then normalized to the baboon's weight (23). By comparison with the total injected activity, the % ID to each organ was determined. Bone marrow activity was derived from blood pool activity according to the model of Siegel *et al.* (24) using a partition fraction of 0.3.

Time-activity curves were generated from the PET ROI results for the seven organs. Each was fit with a biexponential function (Fig. 2) and then integrated numerically to determine the residence time of the activity in each organ. The results accounted for 90–99% of the injected activity over the imaging period, with the rest being included as "missing" and assigned to the MIRD category "remainder-of-body." Thus, the missing fraction was assumed to be distributed uniformly in the body. The "residence time," or accumulation-time product, can be determined for each relevant organ by integrating the time-activity curve either analytically, from 0 to ∞, or numerically over a suitably long interval. We used a numerical integration from 0 to 48 h. The absorbed radiation dose to a given organ was calculated as the sum of the products of the residence times and the tabulated S-values for ^{64}Cu in a standard human geometry. Because the bladder residence time depends primarily on the pattern of voiding, we have calculated a conservative estimate assuming no excretion, as well as a more realistic estimate using the dynamic bladder model available in the MIRDOSE3 software package, with a voiding interval of 4 h.

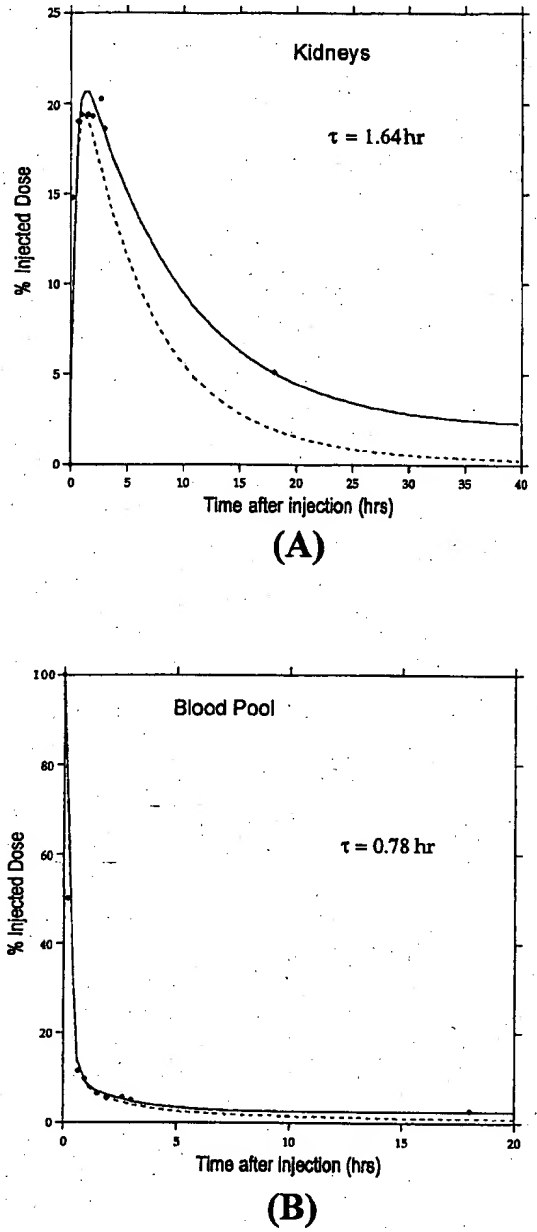

Fig. 2 Time-activity curves for kidneys (*A*) and blood (*B*) from PET images of a baboon injected with ^{64}Cu-TETA-Y3-TATE. Data points in the *upper curve* (*solid line*) are corrected for physical decay, forming the basis for the biexponential fit. The *lower curve* is the fitted curve with physical decay included, from which the residence time, τ, is derived by numerical integration.

Statistical Analysis. To determine statistical significance in the biodistribution studies, a Tukey's Studentized Range (HSD) Test was performed with $P < 0.05$ being consid-

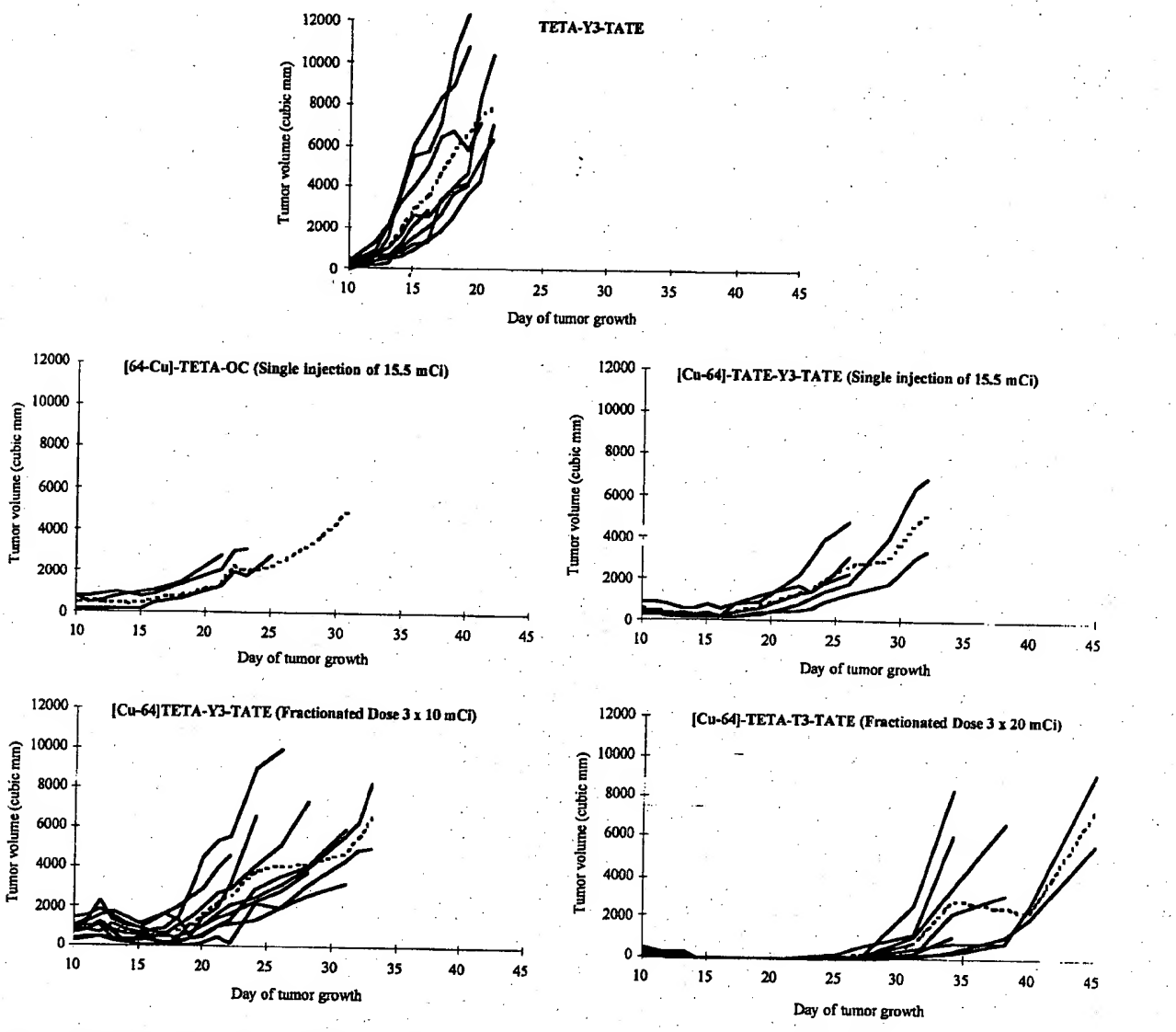

Fig. 3 Radiotherapy experiments in CA20948 tumor-bearing rats. Comparison of tumor growth in control rats and rats treated with ^{64}Cu-TETA-OC and ^{64}Cu-TETA-Y3-TATE. ———, individual rats; ----, mean values.

ered significantly different. In the therapy studies, Tukey's Studentized Range (HSD) Test, ANOVA, Scheffe's test, and a Least Difference Test were performed to compare single-dose and multiple-dose protocols and to compare these with data obtained from control rats. Comparison was made on the length of time it took the tumor to reach 10, 000 mm^3 in size or the time to ulceration.

RESULTS

Radiotherapy Experiments (Single Dose). The results from all of the radiotherapy studies undertaken in CA20948 tumor-bearing rats are shown in Fig. 3. All rats that received a single 15.5-mCi dose of ^{64}Cu-TETA-Y3-TATE showed significant reduction in tumor volume (29–73%) over the following 7-day period, taking 28.4 ± 3.29 days for the tumor volume to reach >10,000 mm^3 or to ulcerate after tumor implant. Inhibition of tumor growth and reduction of tumor size after a single dose of ^{64}Cu-TETA-Y3-TATE were more apparent ($P < 0.05$) than that observed in animals receiving an equal dose of ^{64}Cu-TETA-OC (0–35% reduction over 7 days). There was no tumor growth inhibition in the control groups, and the time it took for the tumors to reach >10,000 mm^3 or to ulcerate (20.71 ± 2.06 days for TETA-Y3-TATE and 19.44 ± 1.67 days for buffer) were significantly lower than the groups that received a single dose of ^{64}Cu-TETA-Y3-TATE ($P < 0.05$). The single 15.5-mCi dose of ^{64}Cu-TETA-Y3-TATE reduced and inhibited the growth of the CA20948 tumor for over 10 days before normal growth resumed. There was no weight loss or appearance of

toxicity with ^{64}Cu-TETA-Y3-TATE at the 15.5-mCi dose. It is important to note that tumors that regrew after remission often ulcerated at an earlier stage than those in controls groups.

Radiotherapy Experiments (Multiple Dose). In both multiple dose experiments, the control groups (10–15 μg of unlabeled TETA-Y3-TATE) showed unrestricted growth of the tumor (mean survival time, 19.25 ± 2.63 days). All rats receiving the 3 × 10 mCi dose regimen of ^{64}Cu-TETA-Y3-TATE showed tumor growth inhibition and a decrease in tumor volume of 36–81%. The smallest tumor sizes were recorded between days 16 and 18 after implantation (0.07–0.60 cm^3). In the treated rats, it took an average of 28.44 ± 3.91 days for the tumors to reach >10,000 mm^3 or to ulcerate after implant. In rats receiving a 3 × 20 mCi dose regimen, there were no palpable tumors in any of the animals at day 14 after tumor implantation. Complete remission and disappearance of the tumor was observed for 10 days. On day 25 after the implant, tumors began to reappear and continued to grow more slowly than before the therapy, until the animals had to be sacrificed 38.22 ± 4.27 days after tumor implant. By using the Tukey's Studentized Range Test, we found that the time it took the tumors to reach >10,000 mm^3 in size or to ulcerate in the rats receiving a 3 × 20 mCi dose regimen was significantly higher than all other groups examined in all other therapy and control protocols ($P < 0.05$).

Blood Chemistry and Physical Appearance. Toxicity was determined in rats receiving 3 × 20 mCi of ^{64}Cu-TETA-Y3-TATE by monitoring weight and gross physical appearance, as well as hematological and liver and kidney function. The mean weight of the treated rats increased similarly to that of the control rats, and they maintained a healthy physical appearance (with no sign of scruffy coat or diarrhea) over the experimental period. Blood chemistries were compared with baseline levels obtained from the control rats. The mean WBC count decreased to 25–50% of the control group level at day 12 after the tumor implant (5,500 ± 3,630/mm^3 *versus* 13,600 ± 1,170/mm^3). The transient drop in WBCs remained constant until day 15 (7,500 ± 1,690/mm^3) and then was seen to recover to baseline levels by day 34 (9,530 ± 2,290/mm^3) after the tumor implant. The differential WBCs showed a transient elevation in segregated neutrophils to a maximum of 407% of controls after 17 days (61.0 ± 5.29% *versus* 15.0 ± 1.63%), with a transient decrease in lymphocytes to a minimum of 45% of controls at day 17 (37.0 ± 4.55% *versus* 82.0 ± 2.16%). Both segregated neutrophils and lymphocyte levels were seen to recover to baseline levels by day 34 (23.75 ± 9.03% and 71.8 ± 8.66%, respectively). No significant changes in RBCs or hemoglobin or hematocrit levels were noted. Platelet levels initially elevated to 138% of controls up to day 17 (11,27.50 ± 106.59 × 10^3/mm^3 *versus* 815.25 ± 87.43 × 10^3/mm^3) and then decreased dramatically to 35% of controls by day 25 (282.25 ± 119.69 × 10^3/mm^3). Baseline platelet levels were reached by day 34 (969.00 ± 151.39 × 10^3/mm^3). The levels of kidney enzymes, blood urea nitrogen and creatinine, did not change significantly over the observation period. Levels of ALP, ALT, and AST were all seen to decrease (65.1, 51.9, and 49.9% of controls, respectively) by day 17. Both ALP and AST recovered to >75% of baseline levels by day 20, and ALP, ALT and AST all returned to baseline levels by day 34.

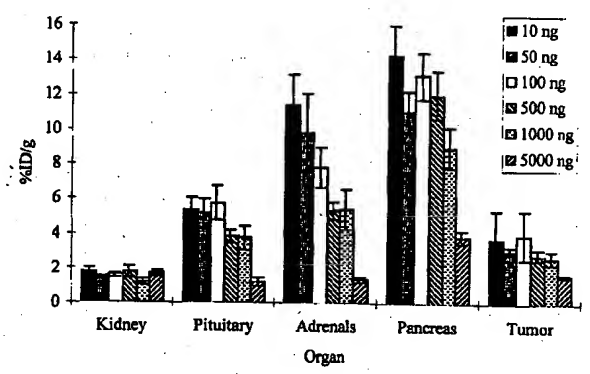

Fig. 4 Biodistribution at 1 h after injection of 5 μCi (0.2 MBq) of ^{64}Cu-TETA-Y3-TATE in CA20948-bearing rats at doses of 10, 50, 100, 500, 1000, and 5000 ng of peptide. *Bars,* SD.

Effect of Specific Activity on Biodistribution of Radiolabeled Peptide. The uptake of 5 μCi (0.2 MBq) of ^{64}Cu-TETA-Y3-TATE, diluted with different masses of unlabeled TETA-Y3-TATE, in receptor-rich organs and the kidney at 1 h after injection is shown in Fig. 4. With a coinjection of 5000 ng of peptide, uptake decreased significantly in receptor-positive tissues ($P < 0.05$). The uptake in the pituitary showed a 4-fold decrease (5.40 ± 0.74% ID/g for 10 ng *versus* 1.24 ± 0.24% ID/g for 5000 ng; $P < 0.05$), the adrenals displayed an 8-fold decrease (11.41 ± 01.67% ID/g for 10 ng *versus* 1.40 ± 0.19% ID/for 5000 ng; $P \leq 0.05$), the uptake in the pancreas was nearly 4-fold lower (14.24 ± 1.74% ID/g for 10 ng *versus* 3.84 ± 0.41% ID/g for 5000 ng; $P < 0.05$), and the tumor uptake was over 2-fold lower (3.69 ± 1.68% ID/g for 10 ng *versus* 1.64 ± 0.06% ID/g for 5000 ng; $P < 0.05$) at the highest mass injected. Other nontarget organs showed no significant decreases in uptake.

Dosimetry. Human absorbed dose estimates to normal organs, calculated from rat biodistribution data and baboon PET imaging, are shown in Table 1. The absorbed dose to the kidneys for ^{64}Cu-TETA-Y3-TATE, based on rat biodistribution, was 0.445 rad/mCi (0.120 mGy/MBq). By comparison, baboon PET image data showed a peak of 20% ID in the kidneys, with fairly rapid clearance, giving an absorbed dose of 1.25 rad/mCi (0.337 mGy/MBq). The intestinal tract also showed distinctly different uptake results from the rat and baboon experiments. There was virtually no uptake in the bowel of the baboon, and only a small fraction (0.9% ID) cleared via the hepatobiliary system. Table 1 also shows the effect of the dynamic bladder model on the dose to the urinary bladder wall in the PET imaging study. In the CA20948 rat model, the average absorbed dose to the tumor was calculated to be 40.8 rad/mCi (11.0 mGy/MBq) for a single injection of ^{64}Cu-TETA-Y3-TATE.

DISCUSSION

^{64}Cu-TETA-OC is presently being evaluated clinically at Washington University School of Medicine for the detection of neuroendocrine cancer by PET (25) and was investigated for therapeutic potential in a rodent tumor model (7). ^{64}Cu-TETA-Y3-TATE has been shown to have a higher affinity for the

Table 1 Absorbed radiation doses resulting from administration of ^{64}Cu-TETA-Y3-TATE, determined from rat biodistribution and PET imaging of a baboon

Organ	Rat rad/mCi (mGy/MBq)	Baboon rad/mCi (mGy/MBq)
Kidneys	0.445 (0.120)	1.25 (0.337)
Liver	0.108 (0.029)	0.39 (0.107)
Gallbladder		0.49 (0.132)
Red marrow	0.069 (0.018)	0.038 (0.009)
Spleen	0.053 (0.014)	0.35 (0.095)
Pancreas	0.145 (0.039)	0.16 (0.043)
Adrenals	0.616 (0.166)	0.059 (0.016)
Upper large intestine wall	0.244 (0.066)	0.035 (0.010)
Small intestine	0.131 (0.035)	0.044 (0.012)
Lower large intestine wall	1.030 (0.278)	0.059 (0.016)
Urinary bladder	0.785 (0.212)	2.82 (0.763)[a]/0.38 (0.103)[b]
Total body	0.040 (0.011)	0.063 (0.017)

[a,b] Urinary bladder dose calculated assuming: [a] no excretion; and [b] using the dynamic bladder model of Cloutier *et al.* (37).

somatostatin receptor than ^{64}Cu-TETA-OC both *in vitro* and *in vivo*; *in vitro*, IC_{50}s for the binding of Cu-TETA-Y3-TATE and Cu-TETA-OC to CA20948 pancreatic tumor cell membranes were 0.250 ± 0.05 nM and 0.498 ± 0.039 nM, respectively; *in vivo*, this increased affinity for somatostatin receptors was shown by a 2-fold uptake of ^{64}Cu-TETA-Y3-TATE over ^{64}Cu-TETA-OC into CA20948 tumors over 1 h (15). The aim of the present study was to determine the radiotherapeutic efficacy of the superior analogue, ^{64}Cu-TETA-Y3-TATE, in a tumor-bearing rodent model, to evaluate the toxicity of the agent, and to calculate human absorbed doses from both rodent biodistribution and primate imaging. The results obtained in this investigation strongly suggest that ^{64}Cu-TETA-Y3-TATE may be superior to ^{64}Cu-TETA-OC and has potential for targeted radiotherapy of neuroendocrine cancer in humans.

From the single-dose radiotherapy experiment (1 × 15.5 mCi), it is evident that ^{64}Cu-TETA-Y3-TATE is at least as effective as ^{64}Cu-TETA-OC in effecting greater tumor regression and may possibly be more effective. In the first multiple dose protocol using ^{64}Cu-TETA-Y3-TATE (3 × 10 mCi), the tumor burden decreased dramatically with an extended time for the tumor burden to reach >10,000 mm^3 or ulcerate in the treated animals compared with the control groups. In the second multiple dose regimen (3 × 20 mCi), there were no palpable tumors in the treated group for an extended period of time (~10 days). Moreover, the time for the tumor burden to reach >10,000 mm^3 or to ulcerate in these rats was nearly twice that of the control groups ($P < 0.05$). All of the statistical analysis performed confirmed that the survival time of the rodents was dependent on the dose administered. It was shown that the 3 × 20 mCi multiple dose regimen was more effective in tumor regression than single-dose administration and the first multiple dose protocol (3 × 10 mCi). The advantages of multiple dose protocols over a single dose have precedence in radioimmunotherapy studies (26, 27) and radiotherapy with peptides (7, 28) and include significant reduction of the tumor burden with decreased toxicity. A multiple dose regimen also has the advantage of delivering a consistent amount of tolerable radiation over an extended period to the tumor, while allowing intermittent recovery of nontarget tissues. The decreased toxicity is often attributable to decreased bone marrow suppression, which is the result of delivery of multiple smaller radiation doses over an extended treatment period.

The CA20948 rat pancreatic tumor is extremely aggressive, with a doubling time of 12–36 h. Stolz *et al.* (4) recently reported complete eradication of CA20948 tumors in 71% of rats treated with 10 mCi/kg (370 MBq/kg) of ^{90}Y-SMT 487 (^{90}Y-DOTA-Y3-OC), with no observable side effects. No absorbed dose measurements to the tumor or normal organs or specific blood chemistry/toxicity data were reported, however. The efficacy of ^{90}Y-SMT 487 has been reported in three patients (11), and this agent is presently in Phase I clinical trials in patients with somatostatin receptor-positive malignancies (3, 29). ^{90}Y has a mean β energy of 0.9 MeV with a maximum energy of 2.27 MeV and a maximum particle range of about 11 mm in tissue, making it an appropriate radionuclide for large tumor burdens. ^{64}Cu emits a 0.58-MeV β^- particle (40%), a 0.66-MeV β^+ particle (19%), and a γ photon of 1.34 MeV (0.5%), yielding a mean range of penetrating radiation of ~1.4 mm in tissue; therefore, ^{64}Cu emissions are more suitable for smaller tumor masses. In the ^{90}Y study, the tumor sizes were 12,805 ± 1140 mm^3 at the time of injection (4). In our studies, rats are sacrificed when the tumor reaches >10,000 mm^3 or the tumor ulcerates; thus, the tumors in our experiments were initially much smaller than those in the investigation of Stolz *et al.* (4). The size of the tumor at the beginning of the treatment may account for the difference in the response of the tumors to the different radionuclides; therefore, a meaningful comparison cannot be made between the efficacy of the ^{90}Y and ^{64}Cu compounds at this time. Moreover, the treatment of the tumor with ^{64}Cu-TETA-Y3-TATE may lead to the selective killing of receptor-rich cells. Theoretically, multiple dose schedules may cause a significant decrease in somatostatin receptor density after repeated administrations. As a consequence, regrowth of CA20948 tumors after treatment is possible because cells with a smaller number or no somatostatin receptors survive during ^{64}Cu-TETA-Y3-TATE treatment. Therefore, because of the longer mean range of the ^{90}Y β^- particle, receptor-negative bystanding cells could also be killed. This may also account, in part, for the complete eradication of CA20948 tumors reported by Stolz *et al.* (4).

In the 3 × 20 mCi dose study reported here, the treated rats gained weight throughout the experiment and at no time presented with any overt physical signs of toxicity, such as lethargy, scruffy coat, >10% weight loss, or diarrhea. Transient elevation and decrease in certain hematological and enzyme levels were noted, but by day 34 after the first treatment, all levels returned to baseline values. Although not fully comprehensive, these toxicity data are encouraging in that a maximum tolerated dose was not achieved and that larger quantities of radioactivity could be administered safely.

The biodistribution of ^{64}Cu-TETA-Y3-TATE in rats was clearly affected by the mass of peptide injected. A bell-shaped relationship has been reported between mass of ^{111}In-pentetreotide and its uptake in receptor-rich tissues (30). In this investigation, there was maximum uptake in somatostatin receptor-positive tissues at the lowest mass dose (10 ng), with de-

creasing uptakes in these tissues at higher masses. For the specific activities given in this report (1.25–2.5 mCi/μg), this would convert to 4–16 μg of material being injected in the 10- and 20-mCi treatment doses. This would result in the lowering of uptake of ^{64}Cu-TETA-Y3-TATE into receptor-rich tissues, which would have direct consequence on the regression of the tumor and dosimetry estimations. Using ^{64}Cu-TETA-Y3-TATE labeled at high specific activities may improve is therapeutic efficacy.

On the basis of the estimated absorbed doses from the baboon PET images, a typical injectate of ^{64}Cu-TETA-Y3-TATE for an imaging study will result in a total dose to the kidneys of 1.25 rad/mCi (0.337 mGy/MBq). This appears to be the dose-limiting organ, because the bladder dose measured in a baboon would be reduced >7-fold by a normal voiding scheme. The kidney dose determined from the nonhuman primate is ~3-fold higher than what was determined from rats. Dosimetry data presented for the intestinal tract also showed distinctly different results from the rat and baboon experiments. The different biodistribution between nonhuman primates and rodents is not surprising, given that hepatobiliary and renal clearance of many radiopharmaceuticals vary widely from rodents to mammals (31, 32). The decreased intestinal uptake and increase of renal dose in the baboon is likely to be more representative of human biodistribution.

Although the large discrepancies between rodent and primate biodistributions of radiopharmaceuticals are not surprising, the fact that human absorbed dose estimates of ^{64}Cu-TETA-Y3-TATE based on rat biodistribution data are greatly underestimated compared with doses obtained from baboon PET imaging data are problematic. Previous studies in our group on the dosimetry of ^{64}Cu-labeled monoclonal antibody 1A3-F(ab')$_2$ showed that absorbed dose estimates from rat biodistribution data overestimated what was found from baboon PET imaging data (33). Preliminary studies of ^{64}Cu-TETA-OC in patients showed that absorbed dose estimates based on rat biodistribution data were not greatly different from actual absorbed doses determined from human PET images.[4] Because of the distinct differences in the biodistribution of ^{64}Cu-TETA-Y3-TATE in rodents and primates, we will base future dosimetry estimates on radiolabeled somatostatin analogues from primate data prior to human studies.

Although human absorbed doses were not estimated in the radiotherapy studies reported previously using ^{188}Re and ^{90}Y-labeled somatostatin analogues in tumor-bearing mice (28, 34), dosimetry results have been reported in the two of the clinical case studies of ^{111}In-DTPA-octreotide therapy. In the report by Krenning *et al.* (8), the patient received a total of 550 mCi (20.4 GBq) over seven administrations, and the estimated doses to the liver and kidneys were 240 and 500 rad (2.4 and 5.0 Gy), respectively, whereas the estimated dose to the tumor was 1300 rad (13 Gy). Fjälling *et al.* (9) reported doses of 630 rads (6.3 Gy) to the liver (which had liver metastases), 371 rad (3.71 Gy) to the spleen, and 212 rad (2.12 Gy) to the kidney and red marrow. A study by Cremonesi *et al.* (35) used ^{111}In-DOTA-Y3-OC to estimate the absorbed doses that would be received in a therapy study with ^{90}Y-DOTA-Y3-OC. They reported the average estimated dose that would be given for a ^{90}Y therapy trial where 30 mCi (1.1 GBq) was administered per cycle for three cycles. The estimated absorbed doses due to ^{90}Y-DOTA-Y3-OC were 231 rads (2.31 Gy) to the liver, 2508 rad (25.08 Gy) to the spleen, 1089 rad (10.89 Gy) to the kidney, 9 rad (0.09 Gy) to red marrow, and 3333 rad (33.33 Gy) to the tumor. In the present study reported here, a single dose of 15.5 mCi of ^{64}Cu-TETA-Y3-TATE was administered to tumor-bearing rats, which weighed ~250 g. Extrapolating this to humans would suggest a total dose of about 4000 mCi (148 GBq) of ^{64}Cu-TETA-Y3-TATE for clinical therapy trials. On the basis of the nonhuman primate data, a delivered dose of 4000 mCi (148 GBq) of ^{64}Cu-TETA-Y3-TATE would result in absorbed doses of 5000 rad (50 Gy) to the kidney, 1560 rad (15.60 Gy) to the liver, 1400 rad (14 Gy) to the spleen, and 152 rad (1.52 Gy) to the red marrow. The kidneys are the critical organ in this study, and a reduction in kidney absorbed dose would be necessary. Methods have been used to decrease the uptake of radiolabeled proteins and peptides in the kidneys, in particular after the i.v. administration of D-lysine (13).

The absorbed dose to the CA20948 tumor from ^{64}Cu-TETA-Y3-TATE, calculated from the rat biodistribution, was 40.8 rad/mCi (11.0 mGy/MBq), compared with 30.9 rad/mCi (8.4 mGy/MBq) from ^{64}Cu-TETA-OC (7). It is important to note that this is the dose to the rat tumor and not an estimated dose to human tumors. By simple calculation, this represents a dose of 6120 mGy (612 rad) for ^{64}Cu-TETA-Y3-TATE to the CA20948 tumor in the single-dose experiment (1 × 15.5 mCi).

The data presented here clearly demonstrate that, for targeted radiotherapy with ^{64}Cu-TETA-Y3-TATE, the use of a multiple dose schedule is superior to single injections in terms of efficacy and toxicity. However, the effect of multiple doses on the tumor uptake of ^{64}Cu-TETA-Y3-TATE must be considered for each consecutive injection. Preliminary studies suggest that uptake of ^{64}Cu-TETA-Y3-TATE in receptor-positive organs and the CA20948 tumor decreases for subsequent identical injections given at 48-h intervals (36). In this study, it was shown that tissue uptake was less affected with the longer intervals between administrations (72 h) and suggests that longer dose fractionation protocols may be superior in therapeutic efficacy than 48-h treatment regimens. Future studies in tumor-bearing rats will include the use of MicroPET imaging (Concorde Microsystems, Knoxville, TN) to determine the optimal time interval for multiple dose regimens. PET imaging will enable the calculation of biodistribution data (*i.e.*, dosimetry measurements) and the determination of therapeutic efficacy simultaneously.

In conclusion, ^{64}Cu-TETA-Y3-TATE was effective in causing tumor regression of CA20948 tumors in rats. A multiple dose regimen of ^{64}Cu-TETA-Y3-TATE temporarily eradicated CA20948 tumors, without lethal toxicity to the animal. It is clear that optimization of the radiotherapeutic multiple dose regimen is necessary to improve upon the results presented in this report. The results reported here also showed significant discrepancies between absorbed dose estimates obtained from rat and baboon biodistribution. These data suggest that ^{64}Cu-TETA-Y3-TATE may not be optimal agent for targeted radiotherapy but does,

[4] C. J. Anderson *et al.*, manuscript in preparation.

however, confirm that other ^{64}Cu-labeled somatostatin analogues warrant continued consideration as agents for targeted radiotherapy.

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